DIFFERENTIAL ANALYSIS OF GENE EXPRESSION DURING *IN VITRO* ADIPOGENESIS IN CATTLE USING AN ADIPOSE TISSUE SPECIFIC cDNA MICROARRAY

By

ASITHA T.VASUDEVAN PILLAI

Bachelor Veterinary Sciences and Animal Husbandry
Kerala Agricultural University
Kerala, India
1998

Master of Veterinary Science
Kerala Agricultural University
Kerala, India
2002

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Thesis Approved:

Dr. Udaya DeSilva

Thesis Adviser

Dr. Clinton R. Krehbiel

Dr. Raluca Mateescu

Dr. A. Gordon Emslie

Dean of the Graduate College

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

Introduction

Consumer demand for high and consistent quality beef has lead to significant changes in the U.S. beef production and marketing systems. Currently, the majority of U.S fed cattle are marketed through a grid marketing system (Forristall et al., 2002; Tess, 2002), which rewards/penalizes producers based on the USDA quality and yield grades classification for carcass beef (United States Department of Agriculture, 1997). The amount and distribution of fat (adipose tissue) plays a central role in this classification system. Due to obvious health reasons, meat with excessive external fat (subcutaneous fat) is less appealing to the consumer and has a low grading score. To increase marketability, excessive external fat is usually trimmed off during slaughter at the expense of total carcass yield. This has resulted in a significant loss to the beef industry, and was estimated to be around $111.99 per steer/heifer slaughtered in 1991 (Savell, 2006). However, deposition of fat within the muscle (intramuscular fat/marbling) is a highly desirable carcass trait as it increases the tenderness and eating quality of meat. Marbled meat is graded high and has more value (Tan et al., 2006). In view of all the above information it is clear that the quality and value of beef is largely dependent on adipogenesis.

Adipogenesis is a complex physiologic process influenced by different factors such as genetics, environment, breed, age, sex, nutrition and management practices (Pyatt and Berger, 2005). Genetic factors significantly influence beef carcass composition and quality traits like marbling, fat thickness, tenderness, juiciness and flavor since these traits are moderate to highly heritable. Traditionally, selective breeding of animals have

been used to generate superior progeny exhibiting desirable traits of economical importance. Information on DNA markers and techniques like marker assisted selection along with other pedigree and phenotypic information make it possible to evaluate and exploit the genetic merit of animals (Bindon, 2004; Burrow et al., 2001). In order to achieve this it is essential to gain an in-depth knowledge of the genetic basis of adipogenesis and identify significant molecular factors that influence body fat distribution and deposition. Mammalian adipogenesis at the molecular level has been studied extensively during the last three decades using human and mouse models. Studies on ruminant adipogenesis are limited to very few reports on some key transcription factors and regulators. This review mainly focuses on growth and development of mammalian white adipose tissue and its molecular control.

Adipose Organ

Adipose tissue, commonly called fat, is a specialized connective tissue present in all mammalian organisms and is characterized by the presence of lipid filled adipocytes and associated stromal vascular cells held in a matrix of collagen fibers. Adipose tissue occurs in two different forms in an animal's body: White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT). The major physiological role of WAT is storage of energy in the form of triglycerides (lipids) during periods of nutritional excess and its release during periods of food deprivation. The distinguishing feature of WAT is the presence of a single, large lipid droplet in the cytoplasm (unilocular) of the adipocytes. BAT is characterized by the presence of multiple, small lipid droplets in their cytoplasm (multilocular) (Cinti, 2005; Greenwood and Johnson, 1983). BAT primarily functions as

a thermogenic organ and serves to provide thermal and energy balance through heat production especially in neonatal and hibernating animals (Cannon and Nedergaard, 2004).

With the presence of two major cell types organized in two tissues (WAT and BAT) and the stromal vascular cells, adipose depots inside an animals body forms the adipose organ (Cinti, 2005). Studies conducted during last couple of decades suggest that the adipose organ secretes a variety of molecules that controls energy homeostasis, immune response, myocardial contraction, fertility, reproduction, hemopoiesis vascular hemodynamics, growth and differentiation (Morrison and Farmer, 1999). This review mainly focuses on growth and differentiation of white adipose tissue.

Adipose tissue occurs in multiple dispersed anatomical locations in the body. Based on the anatomical distribution, total body adipose tissue can be grouped under two major classes: subcutaneous and internal. Subcutaneous adipose tissue can be defined as the layer of adipose tissue found between the dermis and the aponeuroses and fasciae of muscles. Internal adipose tissue can be further classified into visceral adipose tissue (within the chest, abdomen and pelvis) and non-visceral internal adipose tissue. The non-visceral internal adipose tissue includes intramuscular adipose tissue, intermuscular adipose tissue, paraosseal adipose tissue, orbital adipose tissue and any other aberrant adipose tissue depots (Hausman et al., 2001; Shen et al., 2003; United States Department of Agriculture, 1997).

Mature adipocytes constitute the major cell population present in adipose tissue. The remaining fraction includes blood cells, endothelial cells, pericytes, adipose precursor cells of varying degree of differentiation including non-lipid filled mesenchymal cells (Ailhaud et al., 1992; Geloen et al., 1989; Hausman et al., 2001). Mature adipocytes show a bimodal distribution of their diameters. Based on this bimodal distribution the adipocytes can be divided into two fractions: classic adipocyte fraction (35-150µm diameter) and a very small adipocyte fraction (8-35µm diameter) (DeMartinis and Francendese, 1982). Eventhough the functional significance of this bimodal distribution is not fully understood, the existing knowledge suggests that the very small adipocyte fraction indicates hyperplastic growth of adult adipose tissue.

Adipogenesis

Development of adipose tissue from its precursor cells is known as adipogenesis. Adipogenesis occurs throughout the life time of an animal. Increase in adipose tissue mass occurs through hyperplasia as well as hypertrophy.

Adipose tissue is considered to be a specialized form of loose connective tissue. All types of connective tissues present in an animal's body arise from the embryonic mesoderm and is characterized by a highly vascular and complex matrix of extracellular fibers (collagen and elastic), amorphous ground substance and a population of connective tissue cells. During early embryonic development, the mesoderm and neuroectoderm give rise to a loose connective tissue network called mesenchyme. This mesenchyme contains star shaped cells which are considered to be the ancestors of the cellular population of adult

connective tissue including the fibroblast, chondroblast, osteoblast, odontoblast, reticular cells and adipoblasts (Goldberg and Raninovitch, 1983). Even though the mesenchymal lineage of adipose tissue was suggested in 1871 by Fleming, convincing evidence supporting the mesenchymal lineage arose only during 1920s and 1930s. There after, adipose tissue has been considered as an independent organ (Greenwood and Johnson, 1983). Adipocytes constitute the major cell population present in adipose tissue. The remaining portion includes blood cells, endothelial cells, pericytes, adipose precursor cells of varying degree of differentiation and interstitial cells/non-lipid filled mesenchymal cells (Ailhaud et al., 1992; Geloen et al., 1989). As explained above, these mesenchymal cells go through a determination step and change to adipoblasts and several other distinct lineages. Adipoblasts also go through commitment and get converted to pre-adipocytes. It is not certain whether the non-lipid filled mesenchymal cells present in prenatal and adult adipose tissue represent both adipoblasts and pre-adipocytes or pre-adipocytes alone (Ailhaud et al., 1992).

Embryonic development

Studies conducted by Slavin (1979) on embryonic and early postnatal adipose tissue development and differentiation in mouse showed that adipocyte precursor cells appeared on developing inguinal fat pad between days 17 to 19 prenatally and on epididymal, mesenteric and mesometrial regions during one to three days postnatally. These light and electron microscopic studies clearly established that adipocytes differentiate from a pool of fibroblast like mesenchymal cells. Embryonic development of WAT has been subsequently studied in a variety of species including human, pig, rat and mouse

(Ailhaud et al., 1992; Gregorie et al., 1998; Novakofski, 2004; Poissonnet et al., 1988). These studies indicated that formation of adipose cells from mesenchymal tissue begins during mid to late gestation. In case of human embryos, clusters of white adipose tissue start appearing earlier, during the second trimester of gestation at various anatomical sites (Ailhaud et al., 1992; Poissonnet et al., 1988). These studies along with others conducted on cattle, indicate that the chronology of WAT appearance during embryonic and postnatal periods depend strictly on the species and the anatomical location (Gregorie et al., 1998; Hood and Allen, 1975; Robelin, 1985; Robelin, 1986).

During initial stages of development, dense clusters of mesenchymal cells appear near large blood vessels. These mesenchymal clusters transform into primitive fat cell clusters and the cluster size gradually increases with fetal age. In both human and pig embryogenesis, this cluster development is accompanied by the development of an extensive network of capillaries (Ailhaud et al., 1992).

Postnatal development

Rodent models (rats and mouse) have been extensively used to study the postnatal development of WAT. Initial works on postnatal development and cellularity of WAT conducted in rats, as well as normal and obese mice, indicated that the number of adipocytes showed a steady increase immediately after birth and continued that pattern until four months of age (Greenwood and Hirsch, 1974; Hirsch and Han, 1969; Lemonnier, 1972). However, after four months, a steady increase in adipose tissue was observed only in obese animals (Lemonnier, 1972). Lemonnier (1972), in his pioneering

work on effect of diet on fat cell number and size, reported that until four months of age adipose tissue growth occurred through an increase in cell number (hyperplasia) and size (hypertrophy) both in normal and obese mouse. In normal adult mouse (5 to 12 months of age) the increase in fat cell mass was associated with an increase in cell size but not in cell number, while in obese mice, there was a steady increase in both cell number and size. The experimental techniques used in a majority of these studies considered only cells which were completely filled with lipid droplets and hence were not accurate enough to detect modest changes in cell number. So these studies could not give a true estimate of the developmental potential of various adipose tissue depots. The hyperplastic development of adipose tissue in adult animals has been studied extensively during the last three decades and results indicate that precursor cells present in mature adipose tissue have life long potential to make new fat cells (Ailhaud et al., 1992).

In vitro models of adipocyte differentiation

Most of what is known about WAT differentiation originated from *in vitro* studies conducted in embryonic and adipose tissue derived primary cell cultures and cell lines. Two classes of cell lines are currently available for studying various aspects of adipose differentiation: (1) Multipotent stem cell lines that have not been committed to the adipocyte lineage (10T1/2, CHEF/18, Balb/c 3T3) and (2) Pre-adipocyte cell lines that have already been committed to the adipocyte lineage (3T3 L1, 3T3-F442A, Ob1771) (Ailhaud et al., 1992; Gregorie et al., 1998; MacDougald and Lane, 1995b; Novakofski, 2004; Otto and Lane, 2005). 3T3 L1 and 3T3-F442A are the most extensively characterized and frequently used models for studying adipocyte biology. Green and

Meuth (1974) observed that a fibroblast clonal line (3T3) established from disaggregated Swiss mouse embryo cells when grown in culture attained confluence and remained viable in a resting stage for long periods. At confluence, few of these cells showed small lipid droplets in their cytoplasm. These findings resulted in a series of experiments to explore the adipogenic potential of 3T3 lines and the development of the most widely used clonal line, 3T3 L1 (Green and Meuth, 1974; Green and Kehinde, 1974; Todaro and Green, 1963).

One of the major disadvantages of these immortalized cell lines is their aneuploid nature. Researchers often had to verify their findings using primary cultures derived from diploid pre-adipocytes (adult stem cells) isolated from adipose tissue and to a limited extend using transgenic animals (Bluher, 2005; Cornelius et al., 1994). Over the past several years, primary pre-adipocyte cultures have been successfully developed from the mature adipose tissue of rat, mouse, human, pig, sheep, cattle and chicken (Bjorntorp et al., 1980; Gregoire et al., 1992; Hausman and Poulos, 2004; Rodriguez et al., 2004; Soret et al., 1999; Torii et al., 1998; Wu et al., 2000). These primary cultures were developed from fibroblast like interstitial cells isolated from the vascular stroma of different adipose depots commonly referred to as the Stromal Vascular Cells (SVCs). As will be explained later in this chapter, these precursor cells originally represent undifferentiated mesenchymal cells (adipoblasts/pre-adipocytes) (Ailhaud et al., 1992).

Irrespective of their nature, *in vitro* differentiated adipocytes display several characteristics similar to adipocytes inside the body. Faithfulness of primary cultures and

cell lines, which are used as model systems to study adipose tissue development, has been demonstrated by *in vivo* cell transplantation studies. When injected into anatomical sites devoid of adipose tissue, 3T3-F442A and adipose tissue derived pre-adipocytes developed into normal fat pads that are histologically indistinguishable from WAT (Ailhaud et al., 1992; Cornelius et al., 1994; Green and Kehinde, 1979; Sottile and Seuwen, 2000). This evidence along with evidence from several biochemical studies suggests that adipocyte differentiation occurs through similar mechanisms *in vitro* and *in vivo* (MacDougald and Lane, 1995b).

Adipocyte differentiation: Sequential events

The key events during adipocyte differentiation were identified exclusively using pre-adipocyte model systems. These sequential events can be divided into four phases:. commitment, growth arrest, mitotic clonal expansion and terminal differentiation.

Commitment

The process of development of specialized cells from the embryonic stem cells is known as differentiation. Differentiating cells show overt changes in cellular biochemistry and function. Before differentiating into specific phenotypes, embryonic stem cells go through a phase of commitment. The commitment process can be divided into two distinct phases: 1) specification and 2) determination. During specification, cells make a developmental choice and follow a particular path of differentiation if left in their normal environment. Cells at this stage can be reversed to the previous stage. Once the cells go through the phase of determination, they can differentiate autonomously even if they are

placed in another region of the embryo. At this stage, the commitment becomes irreversible (Gilbert, 2000). Even though their fate has been restricted to specific lineages, the committed cells can not be phenotypically distinguished from the uncommitted cells.

Stem cells by definition are "population of cells possessing self renewal capacity, long term viability and multi lineage potential" (Zuk et al., 2002). The self renewal and differentiation ability of stem cells vary depending on the stage of development and based on that stage stem cells can be classified into totipotent, pluripotent, multipotent or unipotent. Cells at pre-blastocyst stage of embryonic development are referred to as totipotent stem cells. These cells are unspecialized, but they can transform into any type of cells during development. After the blastocyst stage, cells begin to specialize and stem cells present in the embryo at this stage are referred to as pluripotent. As the embryo grows, differentiation potential of cells gets restricted to few cell lineages and the cells at this stage are commonly designated as multipotent stem cells. Multipotent stem cells usually reside in tissues among already differentiated cells, but they retain their capacity to differentiate into several related lineages (Robert, 2004). It is well established that the neonatal and adult mammalian adipose tissue contains multipotent stem cells and progenitor cells that can differentiate into adipogenic,osteogenic, chondrogenic, myogenic, neurogenic, endothelial, hematopoietic and hepatic lineages (Cousin et al., 2003; Miranville et al., 2004; Seo et al., 2005; Zuk et al., 2002). These cells are popularly known as SVCs. When exposed to appropriate signal, SVCs undergo the determination

phase and get converted to unipotent progenitor cells which in turn differentiate into corresponding cell types.

Even though none of the regulatory mechanisms that commit stem cells to adipocyte lineage have been identified by *in vivo* studies, several recent *in vitro* studies reported that Bone Morphogenic Proteins (BMPs), members of the transforming growth factor type β superfamily, play a significant role in committing the pluripotent embryonic mesenchymal cell lines to adipocyte and osteoblast lineage (Chen et al., 1998; Fux et al., 2004; Sottile and Seuwen, 2000; Tang et al., 2004). Studies have also showed that BMPs co-operate with members of the CCAAT enhancer binding protein (C/EBPs) family and the level of expression of these proteins direct the pluripotent cells to the adipocyte/osteoblast lineage (Fux et al., 2004). Once determined, the pre-adipocyte cells go through a phase of growth arrest and clonal expansion and finally differentiate into mature adipocytes.

Growth arrest

Pre-adipocytes at confluence go through a stage of growth arrest when cells become density inhibited and stay at the G_0/G_1 cell cycle boundary. This growth arrest is considered to be a pre-requisite for subsequent events happening during *in vitro* differentiation (Gregorie et al., 1998; Otto and Lane, 2005). Growth arrested pre-adipocytes express high concentrations of pre-adipocyte factor-1 (Pref-1), a transmembrane epidermal growth factor-like domain- containing-protein that maintains the pre-adipocytes in undifferentiated state (MacDougald and Lane, 1995a; Smas et al.,

1998). The continued inhibition of Pref-1 expression at the beginning and through the terminal phase of differentiation were found to be necessary for the activation of several key adipogenic transcription factors like CEBPα and PPARγ (Smas et al., 1998).The inhibitory effects of Pref-1 on adipogenesis have been confirmed by its constitutive expression in 3T3-L1 pre-adipocytes (Smas and Sul, 1993). Another similar molecular switch that maintains pre-adipocytes in the undifferentiated stage is Wnt signaling (Gregoire, 2001). The Wnt signaling pathway also regulates differentiation of pre-adipocytes through inhibition of CEBPα and PPARγ. Eventhough few other pre-adipocyte associated adipogenic inhibitors have been described, the exact regulatory pathways are yet to be identified.

Mitotic clonal expansion.

To continue through the differentiation program, growth arrested pre-adipocytes must be exposed to an appropriate combination of mitogenic and adipogenic stimuli (Gregorie et al., 1998; Otto and Lane, 2005). Depending on source (species, age, depot) and type (cell lines/primary cultures/ embryonic/adult), pre-adipocyte cultures vary considerably in their responsiveness to external stimuli (inducers) (Gregoire, 2001; Gregorie et al., 1998). For commonly used pre-adipocyte cell lines like 3T3-L1, 3T3-F442A, C3H10T1 *etc*., a standard mixture containing supra-physiological concentrations of insulin, glucocorticoides (Dexamethasone), cAMP elevating agents (Isobutyl Methyl Xanthine) and Fetal Bovine Serum (FBS) has been proven to be ideal. However, for the differentiation of primary cell cultures, in addition to the above mentioned inducers it

may be necessary to include other agents such as fatty acids, prostaglandins, antidiabetic drugs like thiazolidinedions and/or other hormones, depending on species/source of cells.

On exposure to the most suitable combination of inducers, growth arrested cells re-enter cell cycle and undergo few more rounds of cell division, known as the mitotic clonal expansion (Gregoire, 2001; Gregorie et al., 1998; Otto and Lane, 2005). In 3T3-L1 cells, the transient DNA synthesis associated with mitotic clonal expansion starts around 18h after the addition of inducers. At the end of this phase, cells enter a distinct phase in the G1 growth arrest stage called G_D (Scott et al., 1982). Cells at this stage are characterized by their ability to undergo differentiation without DNA synthesis.

Conflicting reports exist regarding the necessity of mitotic clonal expansion prior to terminal differentiation (Entenmann and Hauner, 1996). However, the majority of studies show that blocking cell cycle progression after induction with rapamycin (Yeh et al., 1995) or aphidicolin (Otto and Lane, 2005; Reichert and Eick, 1999) completely prevent mitotic clonal expansion, expression of adipocyte markers and triglyceride accumulation. The most appealing evidence for a need for mitotic clonal expansion prior to terminal differentiation comes from studies on C/EBPβ depletion in 3T3-L1 pre-adipocytes (Tang et al., 2003; Zhang et al., 2004). C/EBPβ is a crucial transcription factor expressed very early during induction (2-4h). This protein reaches peak concentrations around 24h post induction (Tang et al., 2003). It localizes at the centromere at the beginning of clonal expansion and acquires DNA binding activity. At this stage C/EBPβ transcriptionally activates two important adipogenic transcriptional factors C/EBPα and PPARγ (Lane et

al., 1999). During clonal expansion, the expression of C/EBPβ begins to decline and the levels of C/EBPα and PPARγ slowly increase. These transcription factors act as terminators of mitotic clonal expansion (Yang et al., 2007). Disruption of C/EBPβ results in complete prevention of mitotic clonal expansion and adipogenesis indicating that this protein is crucial for both these processes (Tang et al., 2003). In addition to C/EBPs and PPARs, adipocyte determination and differentiation factor/sterol regulatory element binding proteins (ADD/SREBP) family of transcription factors are also expressed very early during adipocyte differentiation (Gregorie et al., 1998) Other proteins expressed during clonal expansion phase include proteins that are mainly associated with the cell cycle progression like cyclins, cyclin dependent kinases, cMyc proteins and E2F family of transcription factors (Gregorie et al., 1998). Accurate steps and events associated with this very early phase of clonal expansion are yet to be fully identified.

Marked changes in cellular morphology of pre-adipocytes from fibroblast-like cells to round adipocytes occur during clonal expansion and the initial phases of adipogenesis. These morphological changes occur as a result of alterations in the extracellular matrix (ECM) components and the rearrangement of the cytoskeletal network. These structural changes are essential for the communication between ECM and the nuclear matrix and acts as a signal for the activation of adipogenic transcription factors and genes necessary for terminal differentiation. During the first 2-3 days of differentiation, a gradual decrease occurs in the biosynthesis of macro and micro elements of the cytoskeleton such as actin, tubulin, vimentin, vinculin, and tropomyosin (Brandes et al., 1993; Gregorie et al., 1998; Rodriguez Fernandez and Ben-Ze'ev, 1989; Smas and Sul, 1995; Spiegelman

and Farmer, 1982). Ninety to 95% decrease in the synthesis of actin and tubulin is considered to be a key characteristic during this time period (Spiegelman and Farmer, 1982). Along with cytoskeletal components, expression of ECM components is also highly regulated during adipocyte differentiation. Initially, the fibronectin rich ECM of the pre-adipocytes gets converted to an adipocyte basal lamina. Consequent to this, fibronectin, collagen type I, type III and integrin expression decreases and the expression of collagen type IV and entactin (protein of basal lamina) increases (Gregorie et al., 1998; Smas and Sul, 1995).

Terminal Differentiation

Cells that enter the distinct growth arrest stage of G_D (Scott et al., 1982) become committed to go trough terminal differentiation and express the adipogenic differentiation markers. During terminal differentiation cells become spherical and start accumulating multiple small lipid droplets in their cytoplasm. Around 5-7 days post differentiation, the smaller droplets coalesce to form 2 or 3 bigger droplets. These droplets almost completely fill the cytoplasm and push the nucleus towards one side. C/EBPα and PPARγ through auto-activation and cross regulation, maintain the cells in terminally differentiated stage. These proteins work synergistically and activate transcription of hundreds of adipose specific genes (Gregorie et al., 1998; Otto and Lane, 2005; Rangwala and Lazar, 2000; Rosen and Spiegelman, 2000). Transcriptional regulation of adipogenesis by these proteins is explained in the next section. Some of the adipose specific genes activated by C/EBPα and PPARγ include adipocyte type fatty acid binding protein, fatty acid translocase, perilipin, lipoprotein lipase, glucose transport protein 4,

glycerol-3-phosphate dehydrogenase, acetyl-CoA oxidase ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase, glycerol-3-phosphate acyl transferase, fatty acid synthase and phosphoenolpyruvate carboxykinase (Ailhaud et al., 1992; Gregorie et al., 1998; MacDougald and Lane, 1995a; Otto and Lane, 2005; Rosen and Spiegelman, 2000).

Transcriptional control of adipogenesis

Complex molecular mechanisms that control adipogenesis are regulated mainly by three groups of transcription factors: peroxisome proliferator-activated receptors (PPARs), CCAAT enhancer binding proteins (C/EBPs) and adipocyte determination and differentiation–dependent factors/sterol regulatory element binding proteins (ADDs/SREBPs) (Debril et al., 2001; Rosen and Spiegelman, 2000). All these factors function synergistically to control adipogenesis *in vivo*. Eventhough these factors can independently induce adipogenesis in cultured pre-adipocytes, their interdependence and co-regulation are indispensable for maintenance of differentiation.

Peroxisome Proliferator Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) comprise of a subfamily of transcription factors that belong to the nuclear hormone receptor super family. Three distinct members of this subfamily have been identified so far: PPARα, PPARγ and PPARδ(Berger and Moller, 2002; Debril et al., 2001; Fajas et al., 2001; Matsusue et al., 2004; Mueller et al., 2002). PPAR α, the first identified PPAR, was cloned from rodent liver and was recognized as a peroxisome proliferating agent and thus the subfamily was

named as PPARs (Debril et al., 2001; Issemann and Green, 1990). Members of PPAR subfamily are characterized by three major structural and functional domains: N-terminal domain (AF-1), DNA binding domain (DBD) and ligand binding domain (LBD) (Debril et al., 2001; Houseknecht et al., 2002). PPARs heterodimerize with another member of the nuclear hormone receptor superfamily, retinoid X receptor (RXR), and control transcription of several target genes involved in a multitude of physiological processes. The DBD of PPARs recognizes two core hexanucleotide repeats on target genes which are spaced by an adenine nucleotide (5'AGGNCA A AGGTCA3'). These recognition motifs on target genes are called PPAR response elements (PPREs) (Desvergne and Wahli, 1999). Various isotypes and isoforms of PPARs are expressed in different tissues and often times these isotypes show patterns of co-expression. Expression patterns of these isotypes have been studied extensively in human, mouse and xenopus to reveal their functional and biological significance. It has been reported that in adult mammals PPARα is expressed at high levels in cells with higher mitochondrial and peroxisomal beta oxidation like cardiomyocytes, cells of the kidney proximal tubules and enterocytes at the top of the intestinal villi (Desvergne and Wahli, 1999; Semple et al., 2006). PPARδ is abundantly expressed in all adult tissues at higher levels as compared to the other two isotypes (Desvergne and Wahli, 1999; Semple et al., 2006). In contrast to PPARα and PPARδ, PPARγ expression is restricted to few tissues including white adipose tissue (WAT), brown adipose tissue(BAT), colon, cecum, spleen, Payer's patches, retina, heart, liver and skeletal muscle. Among these tissues, WAT and BAT were observed to be the major sites of expression (Debril et al., 2001; Desvergne and Wahli, 1999; Semple et al., 2006). Three major isoforms of PPARγ (PPARγ1, γ2 and γ3) have been observed in most

mammalian species generated mainly by alternative promoter usage and alternative splicing (Rosen and Spiegelman, 2000). Among the three, PPARγ2 expression is restricted mainly to WAT (Mueller et al., 2002; Rosen and Spiegelman, 2000). Adipose tissue specific expression pattern along with the information from loss of function and gain of function experiments have demonstrated that PPARγ plays a pivotal role in adipose tissue growth and differentiation (Debril et al., 2001; Mueller et al., 2002; Rosen and Spiegelman, 2000).

PPARγ activation, a major initial event during adipocyte proliferation and differentiation, was found to be regulated by numerous factors including phosphorylation, lipophillic ligands, activation/inhibition of coactivators/corepressors (Farmer, 2005; Houseknecht et al., 2002) and adipocyte-specific and other transcription factors. Ligand activation and subsequent recruitment of certain co-activators was suggested to be the most important mode of control among those listed above (Debril et al., 2001; Farmer, 2005; Houseknecht et al., 2002). Fatty acids, eicosanoids, prostaglandins (15-PGJ2) and α1-antitrypsin are the common naturally occurring ligands. Several synthetic ligands of PPARγ have also been identified over the past decade and include anti-diabetic drugs like thiazolidinediones (TZDs-roziglitazone, troglitazone, KRP297 etc.) and non-thiazolidinediones like Isoxazolidinedione (JTT-501, GW-7845, GW-0207 ect.) (reviewed in (Houseknecht et al., 2002)). Eventhough a number of natural and synthetic ligands that can initiate adipogenesis have been identified, a true endogenous ligand or a pathway that can activate the release of a true endogenous ligand is yet to be identified (Houseknecht et al., 2002).

Binding of ligands to the large binding pocket formed by 13 α-helices and a 4 strand β-sheet in the LBD leads to a conformational change in the PPARγ protein (Nolte et al., 1998). This ligand bound PPARγ heterodimerizes with RXR and activates several distinct cofactors in a ligand dependent manner, which initiates the transcription of hundreds of adipose specific genes by binding to the PPREs present on the regulatory region of these target genes (Debril et al., 2001; Houseknecht et al., 2002; Nolte et al., 1998). RXR, eventhough not an adipocyte specific transcription factor, is considered to be one of the key regulators of adipogenesis since it is the obligate heterodimeric partner of PPARγ (Debril et al., 2001). Several important genes involved in fatty acid metabolism, transport and storage carry functional PPREs in their regulatory regions. Few examples include adipocyte type fatty acid binding protein, phosphoenol pyruvate carboxykinase, Acyl CoA synthetase, fatty acid transpoet protein 1, lipoprotein lipase, fatty acid translocase and stearoyl CoA desaturase (Debril et al., 2001; Desvergne and Wahli, 1999). PPARγ can activate the uptake of its own ligands like fatty acids and thus initiate/maintain pre-adipocyte proliferation and differentiation by a positive feed back control involving PPARγ and its target genes (Debril et al., 2001)

PPARγ expression is also regulated by members of the C/EBP and SREBP family of transcription factors. The activation and regulation of PPARγ by C/EBP and SREBP are discussed in the next section.

CCAAT/enhancer binding proteins (C/EBPs)

CCAAT/enhancer binding proteins (C/EBPs) belong to a family of transcription factors that bind to the CCAAT box motif present on several gene promoters. These proteins are characterized by a highly conserved basic leucine zipper (bZIP) DNA binding and dimerization domain (Ramji and Foka, 2002).The basic-amino acid rich portion of the bZIP domain binds to the CCAAT box motif on target genes and the leucine zipper serves as the dimerization domain for the homodimer and heterodeimer formation . Six members of this family, C/EBPα, β, γ, δ, ε and ζ, have been identified so far in several species including human, mouse, rat, bovine, ovine, hamster and chicken (Ramji and Foka, 2002). All members except C/EBPζ can homodimerize/heterodimerize with each other and bind to regulatory regions in the target genes. Eventhough C/EBPζ readily heterodimerizes with all other members using its highly conserved leucine zipper, such heterodimers do not bind to the CCAAT motif. Instead, they bind to a completely different recognition sequence (PuPuPuTGCAAT(A/C)CCC, wher Pu is a purine) on the target genes . Hence, C/EBPζ is considered to be a potent inhibitor of all other CEBPs (Hamm et al., 2001; Ramji and Foka, 2002; Rosen and Spiegelman, 2000; Wu et al., 1996).

Among the various isotypes, C/EBPα, β, δ and ζ play important regulatory roles during adipogenesis. Distinct temporal patterns of expression of CEBPs start with a relatively low level of expression of C/EBPζ in confluent pre-adipocytes. As explained earlier, differentiation of confluent pre-adipocytes can be induced by insulin, glucocorticoides (dexamethasone), cAMP elevating agents (IBMX) and FBS. Following induction, the

cells re-enter the cell cycle and go through few more rounds of mitosis called clonal expansion. At this very early phase (zero to 48 h of differentiation), glucocorticoides and cAMP elevating agents present in the media induce a rapid and transient expression of C/EBPβ and δ (Otto and Lane, 2005). Transcriptional activation of C/EBPβ by cAMP occurs as a result of phosphorylation and activation of a protein named cAMP response element binding protein (CREB) and its binding to the cAMP responsive element-like cis regulatory sequences on C/EBPβ gene (Otto and Lane, 2005). These proteins then set off the expression of two important adipogenic transcription factors: C/EBPα and PPARγ2. Since these proteins are the first to appear in differentiating pre-adipocytes and are implicated in the expression of C/EBPα and PPARγ, they are considered to be the key initial regulators of the differentiation process (Darlington et al., 1998; Ramji and Foka, 2002; Rangwala and Lazar, 2000). As differentiation advances C/EBPβ and δ expression begin to decline and C/EBPα and PPARγ expression slowly increases and at the end of mitotic clonal expansion C/EBPα reaches its peak concentration (Lane et al., 1999). Thereafter, C/EBPα and PPARγ stays elevated throughout the entire differentiation program (Lane et al., 1999; Ramji and Foka, 2002). C/EBPα and PPARγ maintain high concentrations through the entire differentiation process by auto-activation and cross-activation of each other's expression through a positive feedback loop. Subsequent to their peak expression, each of these proteins independently activates hundreds of adipose-specific genes and governs the cascade leading to terminal differentiation (Hamm et al., 2001; Lane et al., 1999; Otto and Lane, 2005; Ramji and Foka, 2002).

Another expression event in this sequence is the expression of C/EBPζ, which is expressed in lower concentrations in pre-adipocytes and subsequently goes down to undetectable levels as the differentiation starts (0h) (Darlington et al., 1998; Rosen and Spiegelman, 2000). However, its expression returns to relatively higher levels four to five days after the onset of differentiation and stays elevated thereafter. This expression pattern along with the inhibitory effects observed on adipogenesis following over expression of C/EBPζ in cell culture systems shows that it plays important regulatory roles during adipogenesis (Darlington et al., 1998; Rangwala and Lazar, 2000; Ron and Habener, 1992; Rosen and Spiegelman, 2000)

The role of C/EBPβ and δ in the regulation of C/EBPα and PPARγ activity have been studied extensively (Darlington et al., 1998; Hamm et al., 2001; Lane et al., 1999; Rangwala and Lazar, 2000; Salma et al., 2006; Tanaka et al., 1997; Wu et al., 1996). C/EBPβ and δ directly activate C/EBPα and PPARγ2 through the C/EBP regulatory elements present in the proximal promoters of these genes (Lane et al., 1999) Studies conducted in transgenic mouse and primary pre-adipocyte cultures showed that disruption of C/EBPβ and δ genes prevents the normal development of adipose tissue and differentiation of cultured pre-adipocytes. Eventhough the absence of C/EBPβ and δ affects the normal development and differentiation of adipose tissue, its absence does not severely interfere with the activation of C/EBPα and PPARγ due to the presence of an alternative pathway involving activation of endogenous PPARγ ligads/activators (Hamm et al., 2001; Tanaka et al., 1997).

Sequential localization and binding of C/EBPα, β and δ to the consensus sequences present in the centromere is considered to be another regulatory mechanism associated with the mitotic events occurring during adipocyte differentiation. The first mitotic event, clonal expansion, is a prerequisite for adipocyte differentiation (Gregorie et al., 1998; Otto and Lane, 2005). C/EBPβ and δ, which are not anti-mitotic, localize at the centromere at the beginning of clonal expansion. During the clonal expansion phase, the expression of these proteins begins to decline and the levels of C/EBPα slowly increase. C/EBPα, which is anti-mitotic, reaches its peak concentration when clonal expansion ceases. Thus these three proteins together help the differentiating adipocytes to go through clonal expansion to terminal differentiation (Lane et al., 1999).

Sterol regulatory element binding proteins (SREBPs)

SREBPS are transcription factors that have been established as regulators of cholesterol and fatty acids metabolism. Biological activity of these proteins is controlled by the level of intracellular sterols. These membrane bound proteins are activated by the proteolytic cleavage of the N-terminal residue (Shimano, 2001). Active SREBPs enter cell nucleus and activate transcription of several genes involved in three major pathways involved in lipid metabolism *viz.* cholesterol biosynthesis, fatty acid uptake and cholesterol and fatty acid biosynthesis (Desvergne and Wahli, 1999; Shimano, 2001). SREBPS have been found to play important regulatory roles in adipogenesis by activating two isoforms of PPARγ, PPARγ1 andγ3 (Fajas et al., 1999). SREBPs control PPARγ expression by biding to two distinctive regulatory regions in its promoter, the E-box (CANNTG) and sterol regulatory element (SRE) (Fajas et al., 1999; Kim et al., 1998; Shimano, 2001).

Through the E-box motif, SREBP induces the expression of FAS and LPL, two key genes involved in fatty acid metabolism and its regulation (Fajas et al., 1999; Kim and Spiegelman, 1996). Here SREBP directly induces transcription of PPARγ and in turn the downstream target genes. Its action through SRE is more or less an indirect activation process. During this second process, activated SREBP induces the production of endogenous ligands that can bind and activate PPARγ proteins (Fajas et al., 1999; Kim et al., 1998).

Adipose tissue - Significance in beef industry

Adipose tissue growth and distribution in domestic animals are of great interest for various physiological, commercial and health related reasons. Adipose tissue functions as an energy store which can be mobilized during periods of negative energy balance. Additionally, in a normal growing animal, deposition of lipids indicates the energy needs of the animal and their ability to convert feed into meat.

From a commercial stand point, in many countries including the United States, the amount of total fat accumulated in the carcass serves as a major determinant of its value at the time of slaughter. The United States Official Standards for Carcass Beef amended in 1939 consider fat as a major determinant of the grade description. According to the current grading system, carcass beef standards can be classified into two main grade groups: yield grades and quality grades. The characteristics of the meat, especially marbling and maturity, which predicts the tenderness, juiciness and flavor determines the USDA quality grades (prime, choice, select, standard etc.), while the yield grades (1 to 5)

are determined by the amount of external, kidney, pelvic and heart fat along with the area of ribeye, muscle and the carcass weight (United States Department of Agriculture, 1997). The major functions attributed to the Federal Beef Grading System include, "aid to livestock producers in identifying and receiving prices commensurate with the quality and quantity of the livestock they produce, providing consumers, retailers, and institutions with a uniform supply of meat of the desired quality and assistance in the promotion and marketing of quality products" (United States Department of Agriculture, 2006). The key participants in the chain of beef marketing, producers, packers, wholesalers and retailers, give great importance to the quality and yield grades because the products are either rewarded or discounted based on the amount of marbling and external fat present in the carcass (Wachenheim and Singley, 1999).

According to the latest trends in marketing, the end users/consumers play the most significant part in deciding the position of beef industry in the highly competitive meat environment. Consequently, consumer preferences including demand for high quality beef which is suitable for evolving consumer life styles and price of beef compared to competing meat products like poultry and pork gained the attention of all participants of the meat marketing channel (Wachenheim and Singley, 1999). Over the past three decades (1970-2000) U.S. meat consumption pattern has changed dramatically. Consumers preferred poultry over beef and the per capita beef consumption has declined during this period from 79 to 64 percent (Haley, 2001). Several surveys and studies were conducted during the last 20 years to identify the major factors associated with this dramatic decline in the demand for beef. The results suggested that a combination of

factors rather than a single factor contributes to this significant loss. The most important factor was consumer's preference for meat which is high in quality, healthy, easy to prepare and low in prices compared to other competing products on the market (Schroeder et al., 2000)

During early to mid 1980s major changes in the beef industry occurred because of the finding that consumers are hesitant to buy beef with excessive external fat. In 1990 National Cattlemen's Association announced that the quality defects, excess fat and management problems accounted for a total loss of $5 billion or $192 per slaughtered steer and heifer, of which ~$ 4 billion came from excessive fat alone (Smith et al., 2000). It was necessary to determine the strength of these existing problems to reinstate the beef industry to where it was during 1970s.

The first National Beef Quality Audit (NBQA) was awarded to scientists in 1991 in an effort to assess the quality of beef marketed in the U.S, to identify the vital problems and to develop different strategies to rectify them. The audit report concluded that even though there were great opportunities to reduce the total fat production significantly, "cattle have been fed to heavier weights with approximately the same degree of subcutaneous fat and with less marbling and fewer high quality grades today than in the 1970s" (Lorenzen et al., 1993). The highest level quality grades (U. S. Prime and U. S. Choice) showed a 20% point decline in 17 years, at the same time the yield grade showed only 0.3 point reduction. The audit workshop came up with the following objectives to improve quality: (1) attack waste by reducing excessive external fat (2) enhance taste by

improving overall palatability, tenderness and by assuring sufficient marbling (3) improve management and (4) control weight (Savell, 2006). As per the recommendations of NBQA 1991, beef quality audits have been conducted every five years to review the progress achieved in quality, consistency and competitiveness of beef (Boleman et al., 1998; McKenna et al., 2002). NBQA 1995 identified ten important factors directly contributing to the quality concerns and the majority of them (low overall uniformity and consistency of meat, low overall palatability, insufficient marbling, inadequate tenderness, excessive external and seam fat) were directly or indirectly linked to fat deposition. Goals and strategies were developed by the audit workshop to improve beef quality over the next five years (Boleman et al., 1998). The 2000 audit compared the results of the previous two audits and the results revealed that the industry has accomplished improvements in 'waste and weight but not in taste, tenderness or management' during the period between 1991 and 1995 (McKenna et al., 2002; Smith et al., 2000). First among the eight strategies developed by the 1995 audit to improve quality, consistency, competitiveness and market share of beef was: "assist producers to use selection and management techniques to produce cattle that fit customer expectation for marbling, red meat yield and weight". The third quality audit workshop has shown that the message to improve quality has finally reached at the producer level as indicated by an increase in consumer demand for beef from 1997 through 2000 (Mimert et al., 2002) . Even though the demand showed modest increase, the progress in quality attributes were slow. The challenges identified were analogous to those identified during the second audit. Once again the recognized goals were mostly linked with external and internal fat deposition and its effect on quality (McKenna et al., 2002; Smith et al., 2000).

Eventually, all three national beef quality audits pointed out the need for more research to identify the genetic basis of carcass merit and their application in animal breeding, selection and management.

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CHAPTER II

DIFFERENTIAL ANALYSIS OF GENE EXPRESSION DURING *IN VITRO* ADIPOGENESIS IN CATTLE USING AN ADIPOSE TISSUE SPECIFIC cDNA MICROARRAY

Introduction

Development of adipose tissue from its precursor cells is known as adipogenesis. Adipose tissue in mammalian embryos arises from a pluripotent mesenchymal stem cell population which can differentiate into osteogenic, myogenic, chondrogenic and neurogenic lineages (Cornelius et al., 1994; Ntambi and Young-Cheul, 2000; Zuk et al., 2002). Mesenchymal progenitor cells are also present in the supportive stroma of bone marrow and adipose tissues (Stromal Vascular Cells/SVCs) of adult mammals. It has been reported that these adult stem cells can be easily isolated and used as an alternative source of mesenchymal stem cells to study their lineage specific differentiation (Zuk et al., 2002). Irrespective of their origin, these stem cells get committed to specific lineages *in vitro* on exposure to appropriate chemical and environmental stimuli. Several factors that lead to commitment and differentiation of mesenchymal stem cells to adipose lineage have been identified. These factors include insulin, insulin like growth factor I, cAMP, glucocorticoids, triiodothyronine, growth hormone, prostaglandins, long chain fatty acids, among others (Sottile and Seuwen, 2000). The full complement of factors needed for differentiation into adipocytes vary depending on the type of cell culture and origin of pre-adipocytes (Ailhaud et al., 1992). A combination of insulin, cAMP and glucocorticoid is generally considered necessary for the differentiation of all types of pre-adipocyte cell culture systems (Ailhaud et al., 1992; Sottile and Seuwen, 2000). Progenitor cells when grown to confluence and exposed to the right combination of adipogenic factors go through a stage of post confluent mitosis (clonal expansion), growth arrest and subsequent differentiation (Ntambi and Young-Cheul, 2000). These cellular changes are accompanied by complex changes in expression patterns of

numerous adipogenic transcription factors, signaling molecules, hormones, enzymes and other adipose related proteins (Feve, 2005). Even though the morphological and cellular events appear to be identical between different cell culture systems, the gene expression pattern and activated pathways differ with type of cell culture, species, origin, and culturing protocols. Most of this information on adipose tissue development has been drawn from rodent and human cell culture systems. Ruminants are physiologically and anatomically distinct from monogastric animals. The location and distribution of fat also shows considerable variation. Hence, it is logical to assume that the regulatory mechanisms and gene expression patterns might as well be different in the ruminant species.

Very few studies on gene expression have been so far conducted to identify the key molecular pathways of bovine adipocyte differentiation. The studies were mainly performed to analyze the differential expression of known adipogenic transcription factors like peroxisome proliferator activated receptor gamma and CAAT/enhancer binding proteins during bovine adipogenesis (Wu et al., 2000; Yamada et al., 2007). Microarray technology is an ideal platform to investigate global gene expression profiles especially during cell growth and differentiation. The present study was conducted to understand gene expression changes occurring during bovine pre-adipocyte differentiation. Microarray experiments were conducted on primary cultures of subcutaneous pre-adipocytes using adipose tissue specific cDNA microarrays. One hundred and eleven array elements were found to be differentially expressed with a two fold change in expression in at least one of the time point comparisons.

Materials and Methods

Animals and Tissue collection

Tissue samples were collected from three crossbred steers between 20 and 25 months of age. Subcutaneous adipose tissue was removed aseptically from the neck region immediately after slaughter. Collected tissues were chopped into small pieces and maintained in ice cold Dulbecco's Modified Eagles Medium (DMEM) containing 100IU/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin-B and 500µg/ml gentamycin (transport media) until further processing.

Isolation culture and differentiation of bovine pre-adipocytes

Protocols for the generation of primary cultures and differentiation experiments were adapted with modifications from published literature (Torii et al., 1998; Wu et al., 2000).

A sub-sample of fat tissue was transferred to fresh transport media and finely minced. The tissue was then subjected to an enzymatic disaggregation to disperse the individual cell types. Approximately 10g of finely minced tissue was incubated in DMEM containing 2mg/ml Type II collagenase, 4mg/ml BSA, 20mM HEPES, 100IU/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin-B and 500µg/ml gentamycin (Digestion medida) at 90 rpm and 37°C for 90min. The tissue digest was filtered sequentially through 250 micron and 100 micron filter assemblies to remove undigested tissue particles and other debris. The flow through was collected and centrifuged at 1000 rpm for 10min. The cell pellet containing pre-adipocytes was washed twice with transport

media and then re-suspended in DMEM supplemented with 10% FBS, 100IU/ml penicillin, 100µg/ml streptomycin, and 0.25µg/ml amphotericin-B (Growth Media). The cells were seeded on six well tissue culture plates (Corning, NY, USA) at a concentration of $2.5x10^4$cells/cm^2 in a total volume of 2ml. The seeded plates were incubated in a humidified incubator maintained at a 5% CO_2 atmosphere and 37°C. Cells were supplemented with fresh growth media every 48 h until subsequent sub-culture.
Cells at fourth subculture were grown to confluence and induced with a differentiation media containing DMEM/Ham's F12, 10% FBS, 100IU/ml penicillin, 100µg/ml streptomycin, and 0.25µg/ml amphotericin-B, 10µg/ml insulin, 0.25µM dexamethasone, 0.25mM 3-iso-butyl-1-methylxanthine and 10% lyposin (Abbott Laboratories, Chicago, IL, USA) (induction mediaI). After 48h of differentiation the cells were maintained in induction media devoid of dexamethasone and 3-iso-butyl-1-methylxanthine (induction media II).

Presence of lipid droplets in the cell cytoplasm was confirmed by Oil Red O staining of cells at 120h of differentiation. Oil Red O staining protocol was adapted with modifications from Ramirezzacarias *et al*. (1992). Briefly, following differentiation, media was aspirated from the cell culture plates and cells were washed three times with ice cold phosphate buffered saline solution (PBS). Cells were fixed in formalin for an hour and washed two more times with PBS. A stock solution of Oil Red O was prepared by dissolving 0.35g of stain in 100ml of Isopropyl alcohol. The solution was maintained at room temperature for 24h and filtered through a Whatman # 40 filter paper (Whatman International, Maidstone, England). A working solution of the stain was prepared by

adding 40ml of double distilled water to 60ml of the stock solution (60%). The working solution was placed in the refrigerator at 4°C for 24h and filtered twice using Whatman # 40 filter paper. Staining was accomplished by treating the formalin fixed cells with the working solution of Oil Red O (10ml/75cm^2 flask) for ~2h. After removing the stain, cells were rinsed extensively with double distilled water and air dried. Stained lipid droplets were observed using a phase contrast microscope.

RNA isolation

Three independent cell culture systems, representing three biological replicates, were maintained throughout the study. Total RNA was isolated from cells harvested at seven different time points (6, 12, 24, 48, 72, and 120h) using TRIzol^{TM} reagent (Invitrogen, Carlsbad, CA, USA). Cells in TRizol were taken out from the freezer, thawed on ice and incubated at room temperature for 5min. Cells were then incubated in chloroform (0.2ml/mlof TRizol) for 3min and centrifuged at 4°C for 30min at 9000rpm. The aqueous phase was removed and transferred to a fresh tube. Isopropyl alcohol (0.5ml/ml 0f TRizol) was added to the aqueous phase and maintained at -80°C for one hour followed by centrifugation at 4°C for 15min at 9000rpm. Supernatant was discarded and the RNA pellet was dissolved in 500µl of DEPC treated water. This RNA was then subjected to a second phenol (pH 5.2):chloroform:isoamyl alcohol (25:24:1) extraction and purification. phenol:choroform:isoamyl alcohol mixture was added at 1:1 ratio to the RNA, mixed well and centrifuged at room temperature for 5min at 14,000rpm. The aqueous phase was collected in a fresh tube and RNA was precipitated by the addition of 0.1 volume of 3M sodium acetate and 2.5 volumes of ice cold absolute ethanol. The tubes were maintained

at -80°C for one hour to increase the efficiency of precipitation. The resulting RNA was collected by centrifuging the tubes at 4°C for 30min at 14,000rpm. RNA pellet was re-suspended in 30µl of DEPC treated water are stored at -80°C. RNA quality was assessed by denaturing agarose gel electrophoresis and spectrophotometric analysis.

Adipose tissue specific cDNA microarray

A bovine cDNA library constructed from subcutaneous adipose tissue was used to generate an adipose tissue specific cDNA microarray. The library was sequenced and all the EST sequences were submitted to the GenBank (dbEST IDs and GenBank accession numbers are presented in supplementary table available at http://www.ansi.okstate.edu/faculty/desilva/papers/table.html. In order to annotate the library, the EST sequences were queried against the Bovine Peptide, Unigene, and nr GenBank data base (e^{-15}) respectively. All unknown EST sequences were finally queried against the GenBank nr database (all organisms). A non-redundant set of 1089 clones from the library were selected to include in the array.

PCR-amplified transcripts of selected genes were printed on the array and designated as probes. Array printing procedures were adapted from published literature (Hegde et al., 2000).

The probes re-suspended in 3XSSC were spotted onto Corning® UltraGAPS™ Coated Slides (Corning, NY, USA) using GeneMachines® OmniGrid™ Micorarrayer (Genomic Instrumentation Services, San Carlos, CA). The print head on the microarrayer was

equipped with 16 print tips in a 4X4 fashion. Sub-arrays printed by a single print tip represented a block. Sixteen unique blocks were generated using the 4X4 print tip arrangement. Each sample was replicated twice within a block and each block was printed three times on a single slide to give a final 4X12 array design. This design ensured that each probe was replicated six times (two spots within a block X 3 blocks) in the microarray. The array also contained positive controls, negative controls and normalization controls. A pooled sample of cDNA, used to generate the cDNA library, represented the positive control. Printing buffer alone was used as the negative control. Three plant genes and three genes from the photosynthetic bacteria *Synecocystis* sp. served as normalization controls. The normalization controls were randomly distributed throughout the array. After printing, the slides were left in a humidified atmosphere inside the printing chamber for six to eight hours to facilitate probe attachment on to the slides and generate high quality, uniform spots. The slides were then baked at 80°C overnight and stored in vacuum desiccators at room temperature until hybridization.

The microarray experiment was designed to have three biological replicates. Total RNA isolated from the SVC cultures derived from three individual crossbred steers represented the biological replicates. As explained in previously, the microarrays were fabricated to have six technical replicate spots representing each printed cDNA on a microarray slide. This design provided 18 (3 biological replicates X 6 technical replicates) different data points for final statistical analysis per time point per transcript.

Microarray pre-hybridization

The slides with the immobilized probes were taken out from the desiccator and rinsed in 0.1% SDS for 4-5minutes to remove excess DNA. Slides were then rinsed in sterile ddH2O and incubated in a boiling water bath (97ºC) for 8 minutes to denature the double stranded DNA probes. Using a slidefuge (TeleChem International, Sunnyvale, CA, USA), the slides were dried and placed in a screwcap tube slide holder containing pre-warmed pre-hybridization solution (BlockIt microarray blocking solution TeleChem International, Sunnyvale, CA, USA) and incubated at 49ºC for 4h. Slides were recovered from the pre-hybridization solution, rinsed with ddH2O, dried and kept inside a 49ºC incubator until hybridization.

cDNA synthesis, labeling and hybridization

cDNA synthesis, labeling and hybridization were accomplished using a 3DNA Array 900 indirect labeling kit (Genispere, Hatfield, PA, USA) containing Alexa Flour^{TM}546 (green) and Alexa Flour^{TM}647 (red) fluorophores with few modifications.

For cDNA synthesis, three micrograms of total RNA from the reference and test samples were reverse transcribed in two separate tubes at 42ºC for 4h, using primers provided with the kit and reverse transcriptase (Superscript II RT, Invitrogen, Carlsbad, CA, USA). The primers used were oligo dT sequences with a unique 31mer attached to the 5'end, referred to as the capture sequence. The capture sequence in primers was different for reference and test samples.

The cDNAs thus prepared were then hybridized to the pre-hybridized microarray slides. The 3DNA indirect labeling method involved two sequential hybridization reactions. cDNA from the reference and test samples with unique capture sequences attached at the 3' end was used for the first hybridization. cDNA was mixed with hybridization buffer and applied to the pre-hybridized array under a cover slip, in a total volume of 85µl. These arrays were incubated at 49°C for 18h in a humidified hybridization chamber. Slides were then washed for 15min in 2XSSC/0.1%SDS buffer pre-warmed to 65ºC followed by washes in 2XSSC and 0.2XSSc for 15min each. The slides were finally rinsed with ddH2O and spin dried. In the second hybridization, capture reagent containing the fluorescent labeled complementary oligomer, specific for each cDNA population (reference sample labeled with green fluorophore and the test sample was labeled with red fluorophore) was hybridized to the unique capture sequence attached to the 3' end. The capture reagent was prepared following kit protocol and applied to the array. The array was then incubated at 49ºC for 4h and washed and dried as explained before. The slides were stored in the dark until image acquisition.

Hybridized slides were scanned with ScanArray™ Express confocal laser scanner (PerkinElmer Life Sciences Inc., Bostom., MA). The intensity values for Alexa Flour™546 and Alexa Flour™647 channels for the foreground and background were acquired using GenePix™ Pro 4.0 (Axon Instruments Inc., Union City, CA).

Microarray data analysis

Intensity values generated using GenePix™ Pro 4.0 were analyzed using several packages available in the Bioconductor Software Project (http://www.bioconductor.org/). Intensity estimates of the background and foreground for each spot were generated by GenePix Pro for both red and green channel. Background correction was then performed using Robust Multi-array Average (RMA) algorithm available in the Bioconductor/LIMMA package. After background correction, poor quality spots were removed by filtering. The threshold intensity value was set at 200 and any spots showing intensity of 200 or less in both channels were given low weight and removed from the downstream data analysis. Spots flagged as bad by GenePix were also filtered from further analysis.

Two different kinds of normalization methods were adapted to account for the variation between spots within an array and variation between replicate arrays (biological replicates). The first method, Loess- within print tip group intensity dependent normalization, balanced the effect of variation associated with dye bias, spatial effects and the effect of each print tip group within each array. Quantile normalization was used to balance the effect of red and green intensity bias across arrays (Yang et al., 2002). After data pre-processing and normalization, the expression ratio M for a particular spot/gene (g) was calculated by the formula

$M = \log_2 (R_g/G_{g),}$

where Rg and Gg represented the red and green intensities for gene "g". The M value represented the abundance of genes in the red channel (treatment) compared to the green channel (reference).

In this experiment we used Moderated T-test (t-value) to identify and rank genes which were differentially expressed. List of differentially expressed genes were generated and ranked based on the M value ($\log_2$ ratio) and P-value. Genes with M value ≥ 1.0 or ≤ -1.0, and a P-value <0.01, were considered statistically significantly differentially expressed.

Gene ontology analysis

Gene ontology analysis of the differentially expressed genes was conducted with the help of Genome Function Integrated Discoverer (GFinder), a web tool designed for the gene ontology and pathway analysis of microarray expression available at http://www.medinfopoli.polimi.it/GFINDer/. The gene IDs of the differentially expressed genes were retrieved from the Entrez Gene data base at National Center for Biotechnology Information (NCBI) and were used for the ontology analysis.

Quantitative Real Time PCR assay (qRT PCR)

Expression data generated by microarray experiment was validated using qRT PCR assays. Two up-regulated genes (adipocyte type fatty acid binding protein[FABP4] and fatty acid translocase [FAT]) and one down-regulated gene (epithelial membrane protein 3 [EMP3]) were selected for validation. Gene expression analysis was performed using two step SYBR green qRT PCR. In the first step, cDNA was synthesized from 1.5µg of total RNA in 20µl using random hexamers and reverse transcriptase (Applied

Biosystems, Foster City, CA, USA). Primers for the real time PCR were designed using Primer3 software package (Rozen and Skaletsky, 2000). Primer sequences and the annealing temperatures are presented in Table 1. In the second step, Real Time PCR assay was carried out in 15µl volume in MyiQ Real Time PCR detection system (Bio-Rad Laboratories, Hercules,CA). Each 15µl reaction contained 7.5µl of 2X iQ™ SYBR® Supermix (Bio-Rad Laboratories, Hercules, CA), 400nM forward primer, 400nM reverse primer and 100-200ng of cDNA. Thermal cycling conditions were 95°C for 5min, followed by additional 40 cycles of 95°C for 10sec and 58 to 62°C for 30sec. After the PCR thermal cycling, melt curve analysis was conducted by bringing the reactions to 55°C and increasing the temperature by 0.5°C up to 95°C. 18S ribosomal RNA was used as the house keeping gene for normalization. The assay was conducted as per manufacturer's directions (18 S Ribosomal RNA kit, Eurogentec, Philadelphia, PA, USA). Relative gene expression analysis was performed following the comparative C_T method published by Livak *et a*l. (2001). Threshold cycles (C_T) for the down stream relative expression analysis were set and detected during the geometric amplification phase of each reaction. The ΔC_T value was calculated by subtracting the 18S ribosomal C_T value of each sample from the corresponding gene C_T values [ΔC_T = (Gene ΔC_T)-(18S ΔC_T)]. To calculate the $\Delta\Delta C_T$ value, the highest mean ΔC_T value was taken as an arbitrary constant and was subtracted from all other mean ΔC_T values. Fold change in gene expression was calculated using the formula, Fold Change = $2^{-\Delta\Delta CT}$.

Results

Culture and differentiation of pre-adipocytes

Bovine pre-adipocyte primary cell cultures were successfully established from SVCs isolated from subcutaneous adipose tissue. Cultured SVCs were morphologically similar to fibroblasts. On plating the cells at a concentration of $2.5x10^4$cells/cm^2, confluence was attained in 5 to 6 days (Fig. 1a). Adipocyte differentiation was induced, as described in the materials and methods section, by treatment of confluent cells with induction media I and II. Morphological changes from fibroblast like cells to round adipocytes could be observed starting as early as 12 h post-induction. Small lipid droplets were evident in the cytoplasm following 48 to 72 h of induction (Fig. 1b). The number and size of droplets increased from day two through day five of differentiation, at which time the cytoplasm was almost completely filled with lipid droplets (Fig. 1c). Presence of lipid droplets in the cytoplasm was confirmed by Oil Red O Staining (Fig. 1d).

Adipogenesis was also confirmed at the molecular level based on the expression of fatty acid binding protein 4 (FABP4), a specific adipocyte differentiation marker (MacDougald and Lane, 1995). Relative gene expression changes of FABP 4 at different time points were determined by qRT- PCR analysis. A consistent up-regulation of the gene was evident through out the differentiation process starting from 6h post induction (Fig 2).

Microarray analysis

Bovine cDNA microarrays containing 1089 unique transcripts were used for this study. Relative gene expression levels at six different time points following induction of adipocyte differentiation were determined with respect to the gene expression at 0h of differentiation (confluent pre-adipocytes). One hundred and eleven array elements were found to be differentially expressed with a two fold change in expression in at least one of the time point comparisons. Of the 111 elements, 67 were significantly up-regulated and 44 were significantly down-regulated ($P<0.01$). Twelve genes did not match any of the NCBI data bases used for annotation of the cDNA library. Ten genes were classified under unknown ESTs since there were no annotations associated with them even though they had significant matches. Two of the unknown ESTs were matched to fat cDNA libraries (e-values 0 and 1 e^{-121}, respectively). These two transcripts were consistently up-regulated by more than two fold from 48h of differentiation. The remaining 88 elements corresponded to specific annotated genes in the NCBI database and were used for subsequent gene ontology analysis employing GFinder (http://www.medinfopoli.polimi.it/GFINDer/) and Entrez gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=search&term=).

Functional Categorization

Based on the findings of gene ontology analysis, differentially expressed genes were grouped into 10 functional classes (Fig3). Gene description, dbEST ID, GenBank Accession number, Entrex gene ID and the M-values for these genes are presented in Table 2 and the M values and P values for all the genes on the array are presented in the

supplementary table (http://www.ansi.okstate.edu/faculty/desilva/papers/table.html). Ten genes involved in the metabolism, binding and transport of lipids as well as genes encoding proteins secreted from adipose tissue were grouped together (Fig3.A). Adipocyte type fatty acid binding protein (FABP4), fatty acid translocase (CD36) and Chemokine ligand 16 (CXCL16) were consistently up-regulated after 12h of differentiation. Acetyl CoA synthetase2 (ACSS2), Stearoyl CoA desaturase (SCD), homolog of yeast polyunsaturated fatty acid elongation factor (MGC 128086) and phosphatidic acid phosphatase (PPAP2B), all of which are involved in triacyl glycerol biosynthesis, were up-regulated from 48h through 120h and throughout post-induction. Other genes in this class included apolipoproteins like APOE and serum amyloid A3 (SAA3), and caveolin 1 (CAV1). These three genes were also up-regulated at 48, 72 and 120h time points post induction, respectively.

Differentially expressed enzymes involved in the glycolytic pathways were grouped together (Fig 3.B). Among them, glucose phosphate isometase (GPI), enolase (ENO1) and transketolase (TKT) were found to be up-regulated and phosphoenolpyruvate carboxykinase 2 (PCK2) was found to be down-regulated.

Genes coding for cytoskeletal and extracellular matrix (ECM) proteins were found to be differentially expressed and was most evident between 24 and 72h of adipocyte differentiation (Fig 3.C). Major cytosketal components like beta actin (ACTB), tubulin (TUBB) and vimentin (VIM) were down-regulated starting as early as 24 h post-induction. Gelsolin (ADF), an actin filament depolymerizing factor, was up-regulated at the time points where actin was down-regulated, whereas, microfilament and actin

filament cross linker protein (LOC506730) showed consistent down-regulation throughout the differentiation experiment. ECM components showing fluctuations in expression included four types of collagens (COL1A1, COL3A1, COL4A1 and COL5A2), nidogen (NID1), fibronectin (FN1) and milk fat globule-EGF8 protein (MFGE8). Expression of Types I, III and V collagen were down-regulated from 24 to 72hpost-induction, while Type IV collagen was predominantly up-regulated. NID1 showed a similar pattern of gene expression as COL4A1. Fibronectin (FN) did not show any significant fluctuations in expression during early phases, but was up-regulated in the mature adipocytes at 120h of differentiation. MFGE8, a major cell adhesion protein, was consistently down-regulated throughout differentiation.

Genes involved in cell growth and differentiation were predominantly down-regulated except for cyclin fold protein 1 (MGC 165952) which showed consistent up-regulation from 12h post-induction (Fig. 3D).

Among genes involved in transcription, only high mobility group box protein was down-regulated. All others showed patterns of up-regulation (Fig 3.E).

Oxidoreductases were another important category of genes that showed consistent up-regulation (Fig. 3F). Except for plasma glutathione peroxidase (GPX3) eight of the differentially expressed oxido-reductase genes were up-regulated from 6h through 120h of differentiation. Up-regulation of GPX3 was delayed and started at 72 and 120h post-induction.

Genes involved in translation, protein synthesis and turn over were grouped together (Fig. 3G). Proteases such as peptidase D (PEPD) and leucine amino peptidase 3 (LAP3) were up-regulated and pro-protein convertase subtilisin (PCSK7) was down-regulated. A serine/cysteine proteinase inhibitor Plasminogen activator inhibitor 1 (SERPINE1), was down-regulated from 24h through 120h post-induction. Two chaperon proteins, heat shock 70kDa protein 8 (HSPA8) and C1GALT1-specific chaperone 1(C1GALT1C1), also showed differential expression. HSPA8 was up-regulated during the early time points and C1GALT1C1 was up-regulated from 24 to 120h post-induction. Eukaryotic translation initiation protein 4A isoform1 (E1F4A1) was up-regulated at 6 and 12h post confluence and seryl tRNA synthetase (SARS) was down-regulated from 24 to 72h post induction.

Another group showing differential expression included genes involved in signal transduction (Fig. 3H). Calmodulin 1, bitter taste receptor, RAS like protein rit and protein phosphatase 2 alpha were down-regulated at various time points during differentiation. Genes like Tax1 binding protein, guanine nucleotide binding protein and protein tyrosine phosphatase 4a2 were predominantly up-regulated. Transporters differentially expressed during pre-adipocyte differentiation included ferritin, amino acid transporters, chloride ion transporters and nucleoside transporters (Fig 3I).

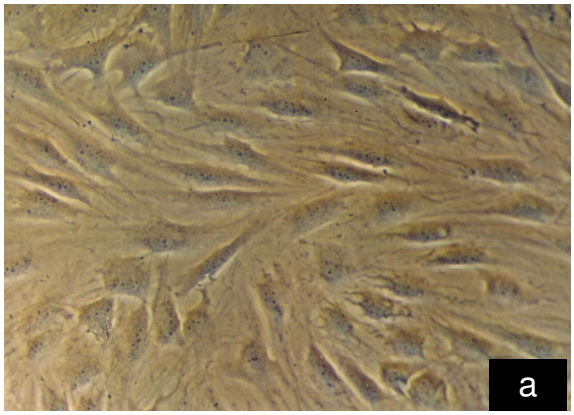
Differentially expressed genes that were not incorporated into any of the above categories were grouped in to a class named ‘others’ (Fig. 3J).

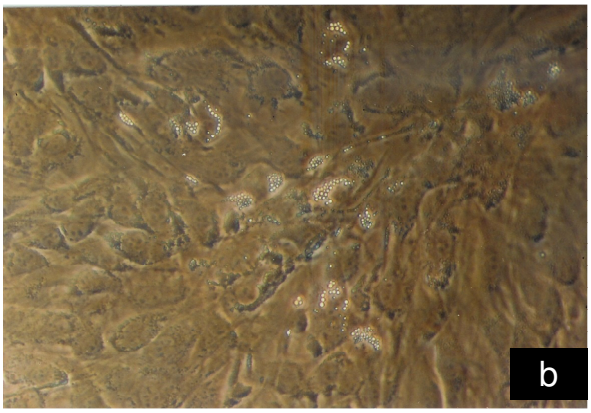
Validation of microarray data using qRT PCR

Three differentially expressed genes were chosen for validation of microarray data. A comparison between the gene expression profiles determined based on microarray and real-time PCR experiments are shown in Figure 4. Adipocyte type fatty acid binding protein and fatty acid translocase that were found to be up-regulated based on microarray data, also showed the same pattern on the real-time PCR experiments. Similarly, epithelial membrane protein 3, which was consistently down-regulated in the microarray experiments, showed a similar trend in real-time PCR experiments.

Figure 1. Microscopic images of Stromal Vascular Cells at different stages of differentiation

(a). Confluent SVCs morphologically similar to fibroblasts (b). Differentiating adipocytes 48h- cells started showing change in morphology by 12h and the lipid droplets were apparent by 48h post induction (c). Differentiating adipocytes 120h post induction- cytoplasm filled with lipid droplets (d) Oil Red O stained lipid droplets in the cytoplasm (120h)


a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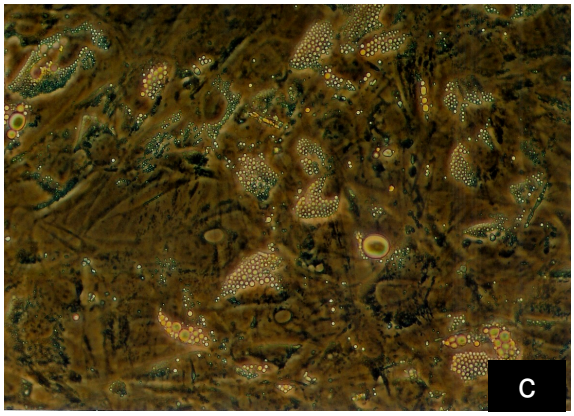
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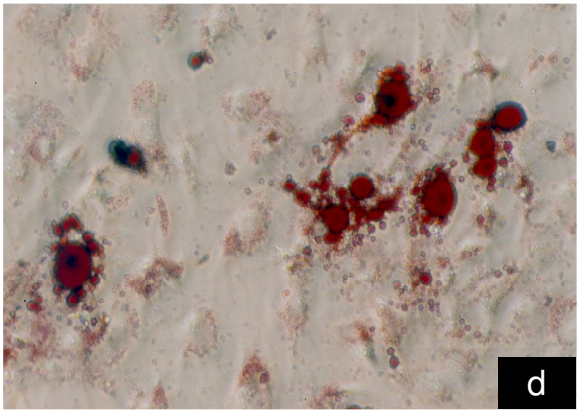

d

Figure 2. Relative changes in the expression pattern of adipocyte type fatty acid binding protein (FABP4) analyzed using real-time PCR.

FABP4 is an early marker of adipocyte differentiation. Its expression increased from 6h through 72h post induction indicating that the cells are differentiating from pre-adipocytes to adipocytes. Bars with different superscripts represent significant differences the fold change in gene expression ($P>0.01$).

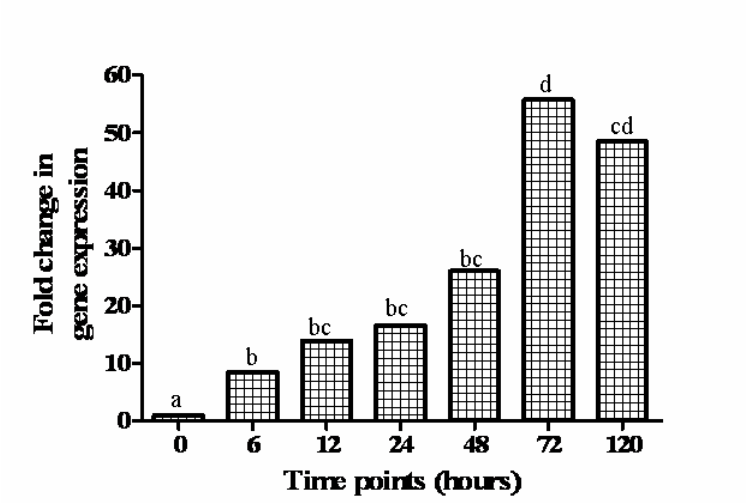
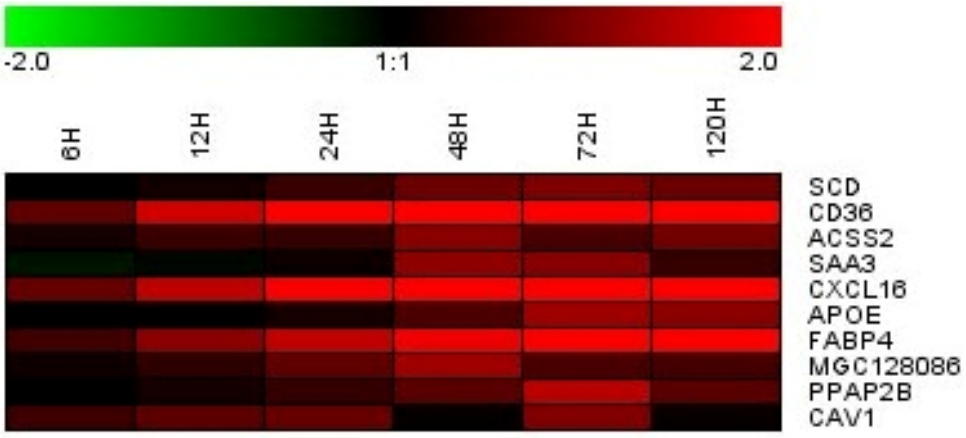

Fold change in gene expression
60
50
40
30
20
10
0
a
b
bc
bc
bc
d
cd
0
6
12
24
48
72
120
Time points (hours)

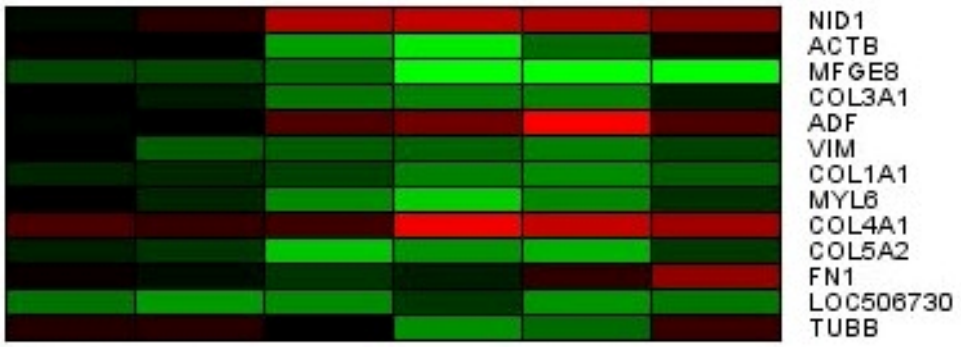
Figure 3. Heat maps showing various gene ontology categories up/down-regulated during bovine pre-adipocyte differentiation

Differentially expressed genes were organized into ten functional categories using GFinder, a web tool designed for the gene ontology and pathway analysis of microarray expression available at http://www.medinfopoli.polimi.it/GFINDer/. These functional categories are presented in Figure 3A through 3J. Gene symbol/Locus ID is used to represent up/down-regulated genes in the heat map. Gene description, dbEST ID, GenBank Accession number, Entrex gene ID and the M-values for these genes are presented in Table 2. Gene expression data for each gene for all the time points (M-values, t-values and P-values) are presented in the Supplementary Table (http://www.ansi.okstate.edu/faculty/desilva/papers/table.html). In the heat map, boxes colored with shades of red represent up-regulated genes and shades of green represent down-regulated genes. Black boxes depict no change in expression.

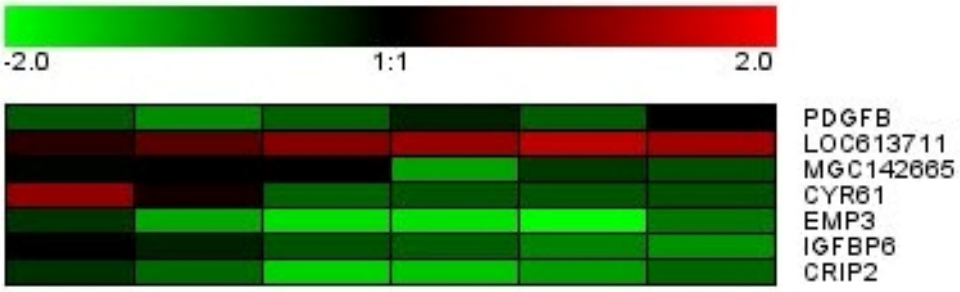


3A. Adipose related

3B. Carbohydrate metabolism



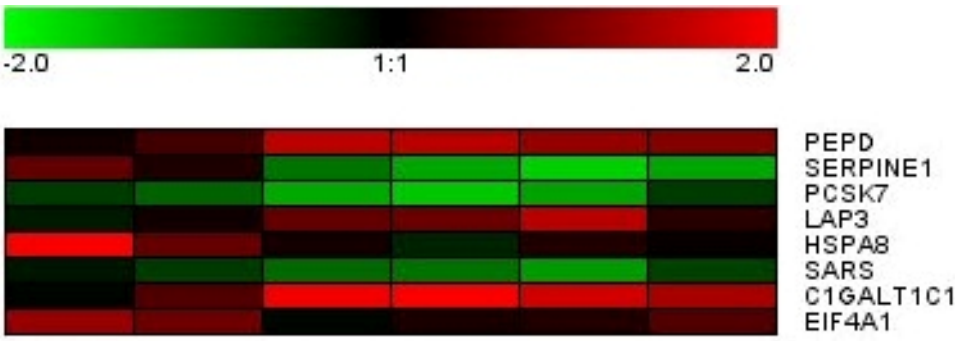
3C. Cytoskeltal/extracellular matrix components



3D. Cell growth/differentiation

3E. Transcription

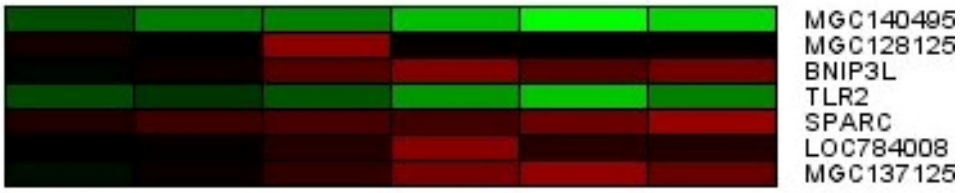
3F. Oxido-reductases



3G. Protein synthesis/turn over

3H. Signal transduction

3I. Transporters



3J. Others

Figure 4. Validation of microarray data on FABP4, CD36 and EMP3 using real-time PCR.analysis.

Three differentially expressed genes (FABP4, CD36 and EMP3) were chosen for validation of microarray data. Graphs on the left show the log ratios obtained from the microarray experiments and the graphs on the right show the relative changes in gene expression observed in real-time PCR. For all three genes presented, fold change in expression was normalized to the highest value. The gene expression patterns in the microarray and real time experiments showed the same trend for all three genes tested.

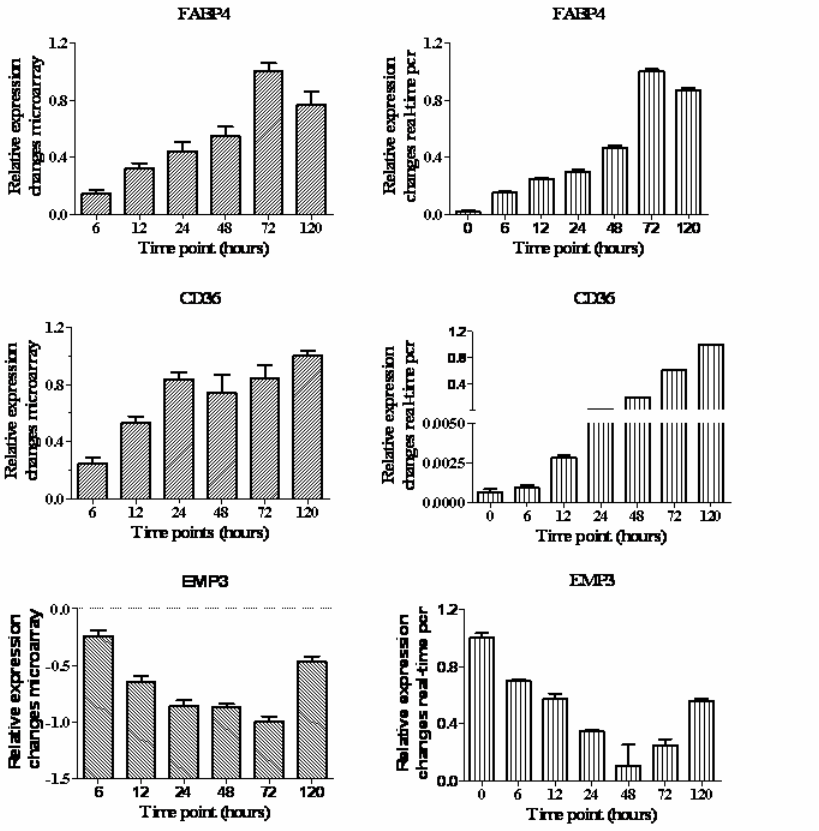

FABP4
Relative expression changes microarray
1.2
0.8
0.4
0.0
6 12 24 48 72 120
Time point (hours)
FABP4
Relative expression changes real-time pcr
1.2
0.8
0.4
0.0
0 6 12 24 48 72 120
Time point (hours)
CD36
Relative expression changes microarray
1.2
0.8
0.4
0.0
6 12 24 48 72 120
Time points (hours)
CD36
Relative expression changes real-time pcr
1.2
0.8
0.4
0.0050
0.0025
0.0000
0 6 12 24 48 72 120
Time point (hours)
EMP3
Relative expression changes microarray
0.0
-0.5
-1.0
-1.5
6 12 24 48 72 120
Time point (hours)
EMP3
Relative expression changes real-time pcr
1.2
0.8
0.4
0.0
0 6 12 24 48 72 120
Time point (hours)

Table 1. Primers used for the real-time PCR analysis

Primer	Sequence	Size (bp)	Tm used	Product Size (bp)	GenBank Accession
FABP4-forward	AAGCTGCACTTCTTTCTCACC	21	62°C	197bp	NM_174314
FABP4-reverse	GACCACACCCCCATTCAAAC	20			
CD36-forward	CAATGGAAAGGACGACATAAG	21	60°C	121bp	NM_174010
CD36-reverse	TGGAAATGAGGCTGCATCTGT	21			
EMP3-forward	TCATCCTCTGCTGTCTGTCCTT	22	60°C	159bp	NM_001024562
EMP3-reverse	CCAGAATCTCTTCGGCATGAAT	22			

Table 2. List of genes which are up/down-regulated during bovine pre-adipocyte differentiation.

Clone ID	Gene Description	dbEST_ID	GenBank Accession	Gene Symbol	Entrez Gene ID	M-Value					
						6H	12H	24H	48H	72H	120H
Adipose related											
g4l23	Acetyl-CoA synthetase 2	46401399	ES345274	ACSS2	506459	0.19	0.47	0.43	1.09	0.57	0.91
g4h03	Apolipoprotein E	46401312	ES345187	APOE	281004	0.00	0.01	0.22	0.62	1.27	1.11
f7b22	Caveolin 1	46400688	ES344563	CAV1	281040	0.49	0.75	0.82	0.07	1.04	0.10
a2f07	CD36 antigen	46400170	ES344045	CD36	518526	0.76	1.67	2.62	2.32	2.65	3.14
g2j05	Chemokine (C-X-C motif) ligand 16	46401209	ES345084	CXCL16	511671	0.81	1.51	1.98	2.26	4.11	3.05
f5e12	Fatty acid binding protein 4, adipocyte	46400286	ES344161	FABP4	281759	0.52	1.12	1.52	1.90	3.48	2.68
a1e11	Homolog of yeast long chain polyunsaturated fatty acid elongatio	46400023	ES343898	MGC128086	617293	0.15	0.38	0.76	1.26	0.59	0.55
f8l24	Phosphatidic acid phosphatase type 2B	46400965	ES344840	PPAP2B	617707	0.02	0.19	0.44	0.72	1.47	0.72
a1g09	Serum amyloid A 3	46400064	ES343939	SAA3	281474	-0.18	-0.10	0.13	1.14	1.08	0.44
a1e12	Stearoyl-CoA desaturase	46400025	ES343900	SCD	280924	-0.02	0.19	0.47	0.87	1.01	0.85
Carbohydrate metabolism											
g1b05	Enolase 1	46401023	ES344898	ENO1	281141	0.24	0.63	1.03	0.92	1.01	0.75
g4p13	Glucose phosphate isomerase	46401454	ES345329	GPI	280808	0.38	1.71	1.78	1.62	1.32	0.97
f6a03	Phosphoenolpyruvate carboxykinase 2	46400519	ES344394	PCK2	282856	-0.40	-0.63	-0.18	-1.23	-0.70	-0.71
a1e09	Transketolase	46400020	ES343895	TKT	445425	0.04	0.35	1.07	0.59	0.99	0.53
Cytoskltal/Extracellular matrix related											
a1h01	Actin, beta	46400070	ES343945	ACTB	280979	0.07	0.03	-1.26	-1.86	-0.84	0.22
f5m18	Collagen alpha 1(III) chain precursor	46400452	ES344327	COL3A1	510833	0.01	-0.23	-0.96	-1.03	-1.03	-0.24
a2a12	Collagen, type I, alpha 1	46400097	ES343972	COL1A1	282187	-0.30	-0.34	-0.53	-1.03	-1.11	-0.74
a1h04	Collagen, type IV, alpha 1	46400074	ES343949	COL4A1	282191	0.59	0.44	0.51	1.91	1.56	1.28
a1g04	Fibronectin 1 isoform 4 preproprotein isoform 5	46400056	ES343931	FN1	280794	0.12	-0.13	-0.42	-0.25	0.38	1.16
f6k21	Gelsolin precursor	46400620	ES344495	ADF	535077	-0.07	0.01	0.64	0.91	2.20	0.61
f5e20	Microfilament and actin filament cross-linker protein	46400294	ES344169	LOC506730	506730	-0.92	-1.23	-1.10	-0.44	-1.18	-0.95
f7g23	Milk fat globule-EGF factor 8 protein	46400729	ES344604	MFGE8	281913	-0.54	-0.57	-0.87	-2.02	-2.34	-2.69

g3c21	Myosin, light chain 6	50172305	EX656782	MYL6	281341	0.02	-0.30	-1.11	-1.61	-1.08	-0.38
g4n11	Nidogen 1	46401421	ES345296	NID1	534319	-0.13	0.33	1.42	1.55	1.39	1.02
f5g22	Procollagen alpha 2(V)	46400341	ES344216	COL5A2	782818	-0.27	-0.42	-1.55	-1.16	-1.41	-0.46
f7k02	Tubulin	46400750	ES344625	TUBB	781412	0.29	0.33	-0.01	-1.14	-0.85	0.47
g1g09	Vimentin	46401065	ES344940	VIM	280955	-0.04	-0.75	-0.74	-0.79	-1.01	-0.53
Cell growth/Differentiation											
f6d15	Cyclin fold protein 1	46400549	ES344424	LOC613711	613711	0.34	0.71	1.06	1.21	1.48	1.28
f7o13	Cyclin-dependent kinases regulatory subunit 1	46400790	ES344665	MGC142665	615827	-0.08	-0.05	-0.03	-1.29	-0.46	-0.63
f8j14	Cysteine-rich angiogenic inducer 61	46400936	ES344811	CYR61	508941	1.16	0.18	-0.79	-0.67	-0.68	-0.64
f7n10	Cysteine-rich protein 2	46400774	ES344649	CRIP2	780821	-0.40	-0.83	-1.68	-1.56	-1.25	-0.82
f5n03	Epithelial membrane protein 3	46400459	ES344334	EMP3	535273	-0.41	-1.33	-1.77	-1.80	-2.06	-0.96
g4j21	Insulin-like growth factor binding protein 6	46401357	ES345232	IGFBP6	404186	-0.05	-0.28	-0.66	-0.74	-1.04	-1.16
g1e16	Platelet-derived growth factor B	46401053	ES344928	PDGFB	540106	-0.72	-1.12	-0.77	-0.28	-0.73	0.02
Transcription											
f7k05	Cellular repressor of E1A-stimulated genes	46400752	ES344627	CREG/	530908	-0.12	0.16	0.47	1.41	2.15	2.39
f5i23	Cyclin L2	46400383	ES344258	CCNL2	538796	0.27	1.28	1.56	2.87	3.90	3.19
g4i12	Forkhead box P1	46401333	ES345208	FOXP1	515903	0.11	0.43	1.07	0.66	0.86	0.36
g4j03	Heterogeneous nuclear ribonucleoprotein F	46401343	ES345218	HNRPF	506917	0.05	-0.08	0.09	0.44	1.48	0.89
f5m03	High mobility group box	46400440	ES344315	LOC782741	782741	-0.28	-0.99	-1.04	-0.72	-1.32	-0.80
f5p23	Prothymosin alpha	46400518	ES344393	PTMA	615626	1.18	0.74	0.07	-0.64	-0.12	-0.01
Oxidoreuctases											
g4d19	Anti-oxidant protein 2 (Peroxoredoxin 6)	46401264	ES345139	AOP2/PRDX6	282438	1.21	1.13	1.17	0.55	0.79	0.64
g1a05	Glutathione peroxidase (GPx) plasma isoform	46401015	ES344890	GPX3	281210	0.16	0.19	-0.14	0.31	1.52	1.50
a1e10	Glutathione S-transferase M1	46400022	ES343897	GSTM1	327709	0.14	0.59	1.40	1.16	1.28	0.92
f6k09	Microsomal glutathione S-transferase 1	46400617	ES344492	MGST1	493719	0.15	0.86	1.48	1.94	2.38	1.67
f5k24	Peroxiredoxin 1	46400418	ES344293	PRDX1	281997	0.83	1.73	2.19	1.71	1.71	2.04
f5l19	Peroxiredoxin 2	46400431	ES344306	PRDX2	286793	0.51	1.22	1.68	0.84	1.24	1.54
g4k22	Thioredoxin	46401378	ES345253	TXN	280950	0.66	1.08	1.20	0.76	1.30	1.70
f7n17	Xanthine oxidoreductase	46400778	ES344653	XDH	280960	1.14	0.97	1.08	0.81	0.75	0.38
Signal transduction											

a2b11	Bitter taste receptor Bota-T2R65A	46400111	ES343986	T2R65A	664647	-0.44	-0.32	-0.47	-0.80	-1.20	-0.84
f8g13	CALM1 protein	46400892	ES344767	LOC786304	786304	-0.27	-0.60	-0.73	-0.86	-1.32	-0.83
f8e06	Guanine nucleotide binding protein 2	46400858	ES344733	GNG2	281203	-0.24	-0.21	1.11	0.24	-0.09	0.18
g3o11	Protein phosphatase 2, catalytic subunit, alpha isoform	50172327	EX656804	PPP2CA	282320	0.04	0.08	0.03	-1.21	0.00	0.08
g2c06	Protein-tyrosine phosphatase, type IVA, member2	46401163	ES345038	PTP4A2	614435	1.34	1.95	2.32	3.20	4.64	3.84
g4g20	Rit	46401307	ES345182	LOC533646	533646	-0.08	-0.13	-1.22	-1.28	-1.29	-0.32
g4l22	T-cell leukemia virus type I binding protein 1	46401398	ES345273	TAX1BP1	535589	0.47	1.15	0.97	0.78	1.23	1.77
Protein synthesis/Turn over											
g4k13	C1GALT1-specific chaperone 1	46401372	ES345247	C1GALT1C1	531644	-0.06	0.64	1.93	2.07	1.66	1.33
g4f06	Eukaryotic translation initiation factor 4A isoform 1	46401285	ES345160	EIF4A1	504958	1.21	0.87	-0.09	0.22	0.28	0.66
f5b17	Heat shock 70kDa protein 8	46400231	ES344106	HSPA8	281831	2.61	0.80	0.20	-0.30	0.28	0.09
f8k22	LAP3 protein	46400952	ES344827	LAP3	281271	-0.23	0.15	0.79	0.84	1.46	0.34
a1d09	Peptidase D	46400005	ES343880	PEPD	615158	0.17	0.53	1.50	1.46	1.20	1.06
g4j17	Plasminogen activator inhibitor	46401354	ES345229	SERPINE1	281375	0.79	0.26	-0.92	-1.32	-1.63	-1.32
g4d21	Proprotein convertase subtilisin/kexin type 7	46401265	ES345140	PCSK7	515398	-0.47	-0.79	-1.35	-1.58	-1.30	-0.47
g4k20	Seryl-tRNA synthetase	46401376	ES345251	SARS	281476	-0.19	-0.47	-0.84	-0.91	-1.23	-0.54
Transporters											
f5i12	Chloride intracellular channel 1	46400379	ES344254	CLIC1	515646	0.41	0.07	-0.22	-1.11	-0.81	-0.29
a2g09	Ferritin, heavy polypeptide 1	46400190	ES344065	FTH1	281173	-0.12	0.44	1.27	1.32	1.21	1.18
f6d16	Ferritin, light polypeptide	46400550	ES344425	FTL	286861	0.26	0.83	1.23	2.01	1.88	2.05
f6c15	Glycoprotein-associated amino acid transporter LAT2	46400534	ES344409	LAT2	536818	0.06	0.40	0.64	1.49	1.44	1.00
f6p20	KIAA0755 protein	46400676	ES344551	SEC24D	504422	-0.10	-0.05	-0.61	-0.71	-0.20	-2.65
a1d10	Solute carrier family 24 member 6	46400007	ES343882	SLC24A6	508887	0.21	0.65	1.49	1.30	1.02	1.10
f8p01	Solute carrier family 29	46401004	ES344879	SLC29A1	510932	0.29	0.17	0.71	0.94	1.66	0.63
a2a10	Solute carrier family 7	46400094	ES343969	SLC7A7	504220	-0.17	-0.42	-0.86	-1.05	-0.80	-0.33
Others											
f8c03	BCL2/adenovirus E1B 19kDa interacting protein 3-like	46400822	ES344697	BNIP3L	534615	-0.06	0.13	0.66	1.03	0.67	0.92
f5n06	Complement factor B precursor	46400462	ES344337	MGC137125	514076	-0.15	0.10	0.40	0.94	1.18	0.82
a2b12	Cytochrome P450	46400113	ES343988	MGC140495	541302	-0.64	-1.00	-1.04	-1.51	-2.17	-1.72

a2f02	F3 protein	46400160	ES344035	LOC784008	784008	0.01	0.07	0.31	1.09	0.32	0.29
f5f05	Proliferating cell nuclear antigen	46400301	ES344176	MGC128125	515499	0.18	0.02	1.14	0.09	0.06	0.05
f8c15	Secreted protein, acidic, cysteine-rich	46400831	ES344706	SPARC	282077	0.28	0.49	0.57	0.52	0.86	1.22
a2a08	Toll-like receptor 2	46400093	ES343968	TLR2	281534	-0.60	-0.42	-0.68	-1.20	-1.57	-1.02

Discussion

In adult mammals, adipose tissue growth occurs through hypertrophy of adipocytes and hyperplasia of mesenchymal progenitor cells present in the vascular stroma. Adipose-derived and embryonic mesenchymal cells from human and mouse have been used as model systems to understand regulation of adipogenesis (Ailhaud et al., 1992; Feve, 2005; Rosen and Spiegelman, 2000; Sottile and Seuwen, 2000). These studies have helped to delineate the process of pre-adipocyte differentiation and its complex regulation at the transcriptional level. The anatomical location and distribution of fat decides the quality and value of the finished product, hence adipogenesis plays an important role in beef production. Even though adipogenesis is well studied in monogastric animals, only limited information is available on adipose tissue development and regulation in ruminants. Information on ruminant and monogastric pre-adipocyte differentiation and metabolic differences between bovine subcutaneous and intramuscular adipose depots indicate substantial species and depot specific differences in the regulatory mechanism that control adipogenesis (Smith et al., 2000). In this study we used bovine subcutaneous pre-adipocyte primary cultures and cDNA microarrays to identify gene expression pattern during bovine *in vitro* adipogenesis.

Out of 1089 transcripts analyzed in this study, 111 transcripts were found to be differentially expressed between pre-adipocytes and adipocytes.

Differentially expressed genes were divided into ten groups based on their molecular function. The first group included genes directly or indirectly associated with adipogenesis, fatty acid metabolism and transport. As expected, these genes were up-

regulated in one or more than one time point studied in this experiment. Expression of genes involved in fatty acid and triglyceride biosynthesis like SCD, ACSS2 and PPAP2B coincided with the appearance of lipid droplets in the cytoplasm of differentiating pre-adipocytes. These findings suggest that triglyceride synthesis starts approximately 2 to 3 days post induction of pre-adipocyte differentiation. SCD is an enzyme that catalyzes the rate limiting step in the synthesis of unsaturated fatty acids from acetyl CoA (Kasturi and Joshi, 1982). It is considered to be a late marker of adipocyte differentiation. Expression of SCD and several other genes involved in adipogenesis have been reported to be regulated by key transcription factors like C/EBP alpha and/or PPAR gamma (Sottile and Seuwen, 2000). PPAR gamma is a member of the family of ligand-activated nuclear hormone receptors and a well known adipogenic transcription factor. In the present study PPAR gamma transcripts did not show differential expression following induction of pre-adipocytes. Similar findings have also been observed during bovine bone marrow derived adipocyte differentiation (Tan et al., 2006). It was suggested to be a result of slightly different transcriptional control mechanisms involved in bovine pre-adipocyte differentiation.

Other adipose tissue specific genes found to be positively regulated by PPAR gamma included CD36 and FABP4. These genes were highly up-regulated as early as 12h post induction and continued to increase up to 72h. FABP4 is considered to be an early marker of differentiation and has been reported to be positively regulated as early as 24h post differentiation in 3T3L1 pre-adipocytes (Burton et al., 2004; Burton and McGehee, 2004; Hansen et al., 2004). However, FABP4 expression during bovine bone marrow derived

pre-adipocyte differentiation has been reported to be negatively regulated at 24h and positively regulated only at 6 days post induction (Tan et al., 2006). This indicates a possible difference in gene expression between pre-adipocytes derived from different anatomical locations. It is important to note that FABP4 polymorphisms are found to be associated with marbling and subcutaneous adipose tissue deposition in cattle and pig (Gerbens et al., 1998; Michal et al., 2006). Comparing pre-adipocytes from different anatomical locations will help to better understand the difference between these fat depots at the cellular and molecular level. CD36 is a membrane bound glycoprotein which regulates up-take of long chain fatty acids by adipocytes and muscle cells. In our experiments, along with FABP4, CD36 also showed an increased expression. Up-regulation of CD36 has not been previously reported during bovine pre-adipocyte differentiation. Studies conducted in 3T3L1 cells showed positive regulation of both FABP4 and CD36 (Yang et al., 2007).

CXCL-16 was another gene found to be up-regulated during adipocyte differentiation. Differential expressions of certain chemokines reported during human pre-adipocyte differentiation indicated a regulatory role of chemokines during adipocyte differentiation (Gerhardt et al., 2001). CXCL16 has two effects; it acts as receptor for oxidized low density lipoproteins in macrophages and also shows chemotactic activity. Both CXCL16 and CD36 have been identified as receptors for lipoproteins in lipid laden macrophages (Minami et al., 2001; Shimaoka et al., 2000). This chemokine was highly up-regulated throughout the process of differentiation. It is possible that CXCL16 along with CD36 served as receptors for the transport of lipoproteins from the media into the cell.

Glycolytic pathway plays and important role in fatty acid biosynthesis by providing substrates and reducing equivalents. Up-regulation of genes involved in glycolytic pathways along with the genes involved in triglyceride synthesis indicates de-novo synthesis of fatty acids and triglycerides in the differentiating pre-adipocytes. During differentiation of 3T3L1 pre-adipocytes, expression of PCK increases during later stages of differentiation and is considered to be a late marker of adipocyte differentiation (Beale and Tishler, 1992). In contrast to 3T3L1 pre-adipocytes, differentiating bovine showed down-regulation of PCK at least during one of the time point comparisons. PCK is the major glyceroneogenic enzyme in adipose tissue which converts pyruvate to glycerophosphate for the synthesis of glycerol for the esterification of fatty acid into triacyl glycerol.

Marked changes in cellular morphology of pre-adipocytes from fibroblast-like cells to round adipocytes is a major initial event occurring during adipogenesis. These morphological changes occur as a result of alterations in the ECM components and the rearrangement of the cytoskeletal network. These structural changes are essential for the communication between ECM and the nuclear matrix and acts as a signal for the activation of adipogenic transcription factors and genes necessary for terminal differentiation (Rodriguez Fernandez and Ben-Ze'ev, 1989; Sottile and Seuwen, 2000). In cell lines, induction results in down regulation of actin, tubulin, vimentin, type I and III collagen, fibronectin, poly-L-Lysine and integrins, where as type IV collagen, entactin and laminins show patterns of up-regulation (Brandes et al., 1993; Rodriguez Fernandez and Ben-Ze'ev, 1989; Sottile and Seuwen, 2000; Spiegelman and Farmer, 1982).

Expression patterns of collagens, actin, tubulin, vimentin and nidogen were comparable to that of other cell lines. However, fibronectin and integrin gene expression in bovine was different from the general pattern reported in other cell lines. Fibronectin has been reported to inhibit differentiation of 3T3L1 cell lines and found to be negatively regulated during differentiation (Rodriguez Fernandez and Ben-Ze'ev, 1989), indicating the importance of fibronectin in 3T3L1 differentiation. In our experiments, fibronectin and integrin were not differentially regulated during early stages of differentiation and fibronectin was up-regulated at 120h of differentiation. Alterations in the expression pattern of ECM and cytoskeltal components have also been reported during the differentiation of bone marrow derived bovine pre-adipocytes. Bone marrow derived bovine pre-adipocytes showed similarities and variations in expression pattern of ECM and cytoskeltal components as compared to adipose derived pre-adipocytes. Taken together all these results indicate the activation of slightly different pathways and regulatory mechanisms based on the source and origin of pre-adipocytes.

Committed pre-adipocytes go through a stage of growth arrest at confluence. They should be exposed to appropriate combinations of mitogenic and adipogenic stimuli in order to continue with subsequent mitotic clonal expansion, a special growth arrest and terminal differentiation phases (Otto and Lane, 2005). Several hormones, cell cycle modulators and mitogenic factors have been reported to be either up or down- regulated during this early stage of differentiation. Expression patterns of these proteins largely depend on the type of primary culture and cell line used (Sottile and Seuwen, 2000). In the present study seven genes involved in cell proliferation and differentiation were found to be

differentially expressed. Six of this *viz*. platelet derived growth factor B (PGDFB), cyclin-dependent kinase regulatory subunit 1 (CKS1), cysteine-rich angiogenic inducer 61 (CYR61), epithelial membrane protein 3 (EMP3), insulin-like growth factor binding protein 6 (IGFBP6) and cysteine rich protein 2 (CRIP2) were down-regulated. IGFs and IGFBPs are considered to be crucial regulators of pre-adipocyte differentiation.

Supraphysiological concentrations of insulin used in the induction media acts through IGF signaling pathway and is involved in the initiation of differentiation (Otto and Lane, 2005; Sottile and Seuwen, 2000). Previous studies showed that IGFBPs can modulate adipocyte differentiation and this could be agonistic or antagonistic (Boney et al., 1994; Sottile and Seuwen, 2000). Similarly, PGDFB also can have either inhibitory, stimulatory or no effect on pre-adipocyte differentiation (Sottile and Seuwen, 2000). CKS1 is a component of the CDC28 protein kinase which promotes mitosis by transcriptional activation of CDC20 (Morris et al., 2003). The down regulation of IGFBP6 along with the other modulators of cell proliferation after 12-24h of induction might be indicative of reduction in cell proliferation and mitogenic activity and initiation of differentiation. The only protein that was up-regulated under this functional category was cyclinY or cyclin fold protein 1, a protein involved in cell cycle progression. This protein was consistently up-regulated from 24h through 120h of differentiation. Very limited information is available regarding the function of this protein and its modulatory effect on cell cycle progression. Since it was highly up-regulated 24h post induction, *i.e*. after the clonal expansion phase, it could be acting as a modulator of other cyclins and mitogens. The gene expression pattern observed for cell cycle related proteins indicates that bovine

adipose derived pre-adipocytes enter the special growth arrest/differentiation between 12-48h of differentiation.

Transcription factors modulating pre-adipocyte differentiation have been studied extensively in a variety of mammalian pre-adipocyte cell culture systems. As mentioned before, PPAR and C/EBP families of transcription factors are factors that modulate adipogenesis in monogastric animals like mouse and human. None of the C/EBP family members were represented on our array. PPAR gamma was not found to be differentially regulated. Similar findings were also reported in bovine bone marrow derived pre-adipocyte differentiation studies (Tan et al., 2006). Ohyama *et al* (1998) reported that PPAR gamma 2 is expressed in bovine adipose tissue and adipose derived pre-adipocyte primary cultures. The expression was reported to be increased during pre-adipocyte differentiation when the cells were induced with an exogenous PPAR gamma ligand.

Cellular repressor of E1A stimulated genes (CREG) is an antagonist of adeno virus E1A protein. Adeno Virus E1A protein is known to act as a transcriptional activator and repressor which promotes cell proliferation and inhibits differentiation. CREG being an antagonist of E1A can act as a controller of cell differentiation (Veal et al., 1998). CREG has been reported to have the ability to rapidly induce differentiation in a variety of cell types including pluripotent mouse EC cells, monocytes, myeloid cells and vascular smooth muscle cells (Han et al., 2007). CREG was positively regulated in our experiments from 48 through 120h of differentiation, and it coincides with the appearance of fat droplets in the cytoplasm of differentiating cells. These findings

indicate that CREG acts as an important modulator of bovine pre-adipocyte differentiation by promoting differentiation. Other transcription associated genes modulated during this differentiation included forkhead box P1 (FOXP1), prothymosin alpha (PTMA), cyclin L2 (CCNL2), and heterogeneous nuclear ribonucleoprotien F (HNRPF). CCNL2 is an RNA polymerase II associated protein which functions as a regulator of pre-mRNA splicing (Yang et al., 2004). The direct mechanism of action of L cyclins (like CCNL2) is not completely elucidated. CCNL2 showed consistent up-regulation starting as early as 12h post induction.

Several oxido-reductases/antioxidant genes represented on the array showed consistent up-regulation during the differentiation process. Being a tissue that accumulates fat droplets in its cytoplasm, adipose tissue is inclined to be exposed to stress associated with lipid peroxidation and reactive oxygen species (ROS). Enzymatic and non-enzymatic cellular antioxidant defenses protect adipose tissue against ROS. Enzymes involved in redox metabolic pathways (associated with ROS neutralization) can modulate proliferation and differentiation. ROS released from mitochodria can act as anti-adipogenic signaling molecules and prevent pre-adipocyte differentiation (Carriere et al., 2003; Carriere et al., 2004; Galinier et al., 2006). Recent reports demonstrating the regulatory role of Xanthine Oxidoreductase (XDH) in 3T3L1 and GPX3 on bovine intramuscular pre-adiocyte differentiation also shows the involvement of antioxidants during pre-adipocyte differentiation (Cheung et al., 2007; Tadashi et al., 2006). It is also important to note that there is substantial difference in the expression pattern of several antioxidants between 3T3L1, human, bovine intramuscular, bovine bone marrow and

bovine subcutaneous pre-adipocyte cells (Tadashi et al., 2006; Tan et al., 2006). This highlights the difference in the regulatory mechanisms associated with pre-adipocyte differentiation among different species and specific fat depots within a species.

In addition to the above mentioned transcripts, several other transcripts involved in signal transduction, protein synthesis/ turn over and transport were also found to be differentially regulated during bovine subcutaneous pre-adipocyte differentiation.

Conclusion

The present study gives an overview of gene expression changes associated with bovine adipose tissue derived pre-adipocyte differentiation. The majority of the differentially expressed genes were grouped under adipose associated, cytoskeletal and extracellular matrix components, oxido-reductases and transporters indicating the importance of the associated pathways in bovine adipogenesis. Differences in the expression pattern of several important genes observed in this study compared to the patterns reported in other cell culture systems indicate the existence of distinct regulatory mechanisms and pathways. More work needs to be conducted using whole genome arrays to obtain a more comprehensive and global pattern of gene expression during bovine adipogenesis.

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VITA

Asitha T. Vasudevan Pillai

Candidate for the Degree of

Master of Science

Thesis: DIFFERENTIAL ANALYSIS OF GENE EXPRESSION DURING *IN VITRO* ADIPOGENESIS IN CATTLE USING AN ADIPOSE TISSUE SPECIFIC cDNA MICROARRAY

Major Field: Animal Science

Education:

Bachelor of Veterinary Sciences and Animal Husbandry, Kerala Agricultural University, Kerala, India, 1992-1998

Master of Veterinary Sciences (Animal Nutrution), Kerala Agricultural University, Kerala, India, 2000-2002

Completed the requirements for the Mater of Science Degree in Animal Science at Oklahoma State University, Stillwater, Oklahoma in May, 2008.

Experience:

Teaching Assistant/Graduate Research Assistant, Department of Animal Science at Oklahoma State University, Stillwater, OK, 2003-2008

Vetereinary Surgeon, Department of Animal Husbandry, Govt. Of Kerala India, 1999-2000 and 2002

Name: Asitha T. Vasudevan Pillai Date of Degree: May, 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DIFFERENTIAL ANALYSIS OF GENE EXPRESSION DURING *IN VITRO* ADIPOGENESIS IN CATTLE USING AN ADIPOSE TISSUE SPECIFIC cDNA MICROARRAY

Pages in Study: 85 Candidate for the Degree of Master of Science

Major Field: Animal Science

Scope and Method of Study:
Mammalian adipogenesis at the molecular level has been studied extensively during the last three decades using human and mouse models. Studies on ruminant adipogenesis are limited to very few reports on some key transcription factors and regulators. The present study was conducted to understand gene expression changes occurring during bovine pre-adipocyte differentiation.

Findings and Conclusions:
Gene expression changes during adipogenesis were studied using adipose tissue specific cDNA microarray and primary cell culture system derived from bovine subcutaneous adipose tissue. Results of the microarray analysis showed that 111 genes were significantly differentially expressed during the differentiation process. Differences in the expression pattern of several important genes observed in this study compared to the patterns reported in other cell culture systems indicate the existence of distinct regulatory mechanisms and pathways

ADVISER'S APPROVAL: Dr. Udaya DeSilva